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The Journal of Biochemistry, Vol. 45, No. 1, 1958

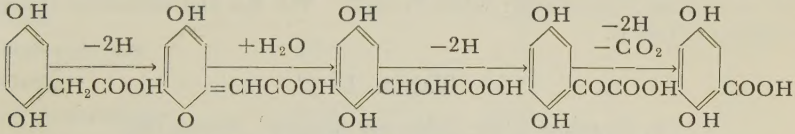
ON THE FORMATION OF GENTISIC ACID FROM HOMOGENTISIC ACID. III*

By YUKIYA SAKAMOTO, TOMOJI MITSUHASHI and KATASHI ICHIHARA

(*From the Biochemical Department, Medical School, Osaka University, Osaka*)

(Received for publication, July 22, 1957)

In the previous papers (*1*, *2*) GA was demonstrated to be formed from HGA by the liver extract of rabbit treated with nitroso-R salt and by that of vitamin C deficient guinea pig and the following scheme was presented (*1*).



In this paper the formation of GA from the assumed intermediary compounds is described in detail.

EXPERIMENTAL

Materials and Methods

Compounds—DPGOA (m.p. 141°) was synthesized by the oxidation of *o*-hydroxyphenylglyoxylic acid according to the method described by Neuberger (*3*), which was obtained from isatinic acid (*4*) or cumaranon (*5*, *6*). DPGCA (m.p. 143°) was obtained by the reduction of DPGOA with natriumamalgam. GAL (m.p. 99°) was synthesized by the procedure of Tieman and Müller (*7*), and HGA (m.p. 147°) was isolated from the urine of an alcaptonuria.

Enzyme Solution—The rabbit was sacrificed by bleeding and the liver was ground with the same amount of sea sand. The paste was extracted with three volume of *M*/10 phosphate buffer (pH 7.2) in the ice box for an hour and centrifuged at 3,000 r.p.m. for 20 minutes. The supernatant was used as the crude enzyme solution.

To the supernatant nitroso-R salt was added at a final concentration of 10^{-3} *M* and the protein precipitating between 50 to 70 per cent saturation with ammonium sulfate was taken (see Table III). It was dissolved in a small volume of water, then adsorbed to two volumes of 2 per cent Ca-gel (pH 6.2). After an hour the mixture was centrifuged and the enzyme was eluted from Ca-gel with 5 ml. of *M*/10 phosphate buffer (pH 8.0) for 10 hours (adsorbed enzyme).

The supernatant from the Ca-gel, was adjusted to pH 5.0 by the addition of acetic acid

* This work is supported by a research grant from the Department of Education.

Abbreviation: GA: gentisic acid, GAL: gentisic aldehyde, HGA: homogentisic acid, DPGOA: 2, 5-dihydroxyphenylglyoxylic acid, DPGCA: 2, 5-dihydroxyphenylglycolic acid, HPAA: *o*-hydroxyphenylacetic acid, SA: salicylic acid.

and centrifuged. The precipitate was dissolved in the same volume of $M/10$ phosphate buffer (pH 7.0) to use for the experiment (isoelectric enzyme).

Reaction Conditions—The enzyme reaction was carried out under the following condition using Warburg's manometer;

Main chamber	1 M phosphate buffer (pH 7.4)	0.3 ml.
	Enzyme preparation	1.0 ml.
	Addition (final concentration	10^{-3} M)
	Total volume was made to 2.5 ml. by the addition of water.	
Side arm	Substrate (2 μM)	0.5 ml.
Center well	50 per cent KOH	0.1 ml.
	The reaction was carried at 37.5° for 2 hours.	

Paperchromatography—After incubation 1.0 ml. of 25 per cent H_2SO_4 was added and filtered. The filtrate was twice extracted with two volumes of ether which was then evaporated. The resulting sirup was dissolved in 0.2 ml. of water and subjected to the paperchromatography with the following solvent systems, butanol: acetic acid: water (4:1:5) or benzene: acetic acid: water (4:5:1). The R_fs and maximum absorptions are as follows (Table I).

TABLE I

The Comparison of R_f Values and Absorption Maxima (mμ) *of Various Compounds*

Developing solution	HGA	GA	GAL	DPGOA	DPGCA
Butanol: acetic acid: water	0.80	0.85	0.95	0.48	0.62
Benzene: acetic acid: water	0.35	0.45	0.85	0.28	0.17
Beckman spec.* absorption maximum (mμ)	290	318	255 350	260 360	290

* Substrate was dissolved in $M/10$ phosphate buffer (pH 7.2).

I. Degradation of HGA by the Liver Extract of Rabbit in the Presence of Metalcatchers

2 μM of HGA was added to 1.0 ml. of the crude liver extract of rabbit in the presence of added nitroso-R salt, KCN, NaN_3, cupferron, diethyldithiocarbamate, EDTA, *o*-phenanthroline, α, α'-dipyridyl, 2-thiouracil, xanthurenic acid or kynurenic acid respectively at a final concentration of 10^{-3} M. After 2 hours incubation paperchromatographies were carried out as described above.

Among the metalcatchers tested, in the presence of nitroso-R salt, oxine and *o*-phenanthroline four more yellow fluorescent spots beside the fluorescent spot of GA (R_f 0.85) were detected on the paper with ultraviolet light, R_f 0.95, 0.77, 0.61 and 0.48. In the case of nitroso-R salt the spot of GA was most strong, while other two compounds at R_f 0.61 and 0.48 were stronger in the cases of oxine and *o*-phenanthroline.

Among four spots described above, GAL (R_f 0.95) DPGCA (R_f 0.61)

and DPGOA (R_f 0.48) were identified by comparing with the authentic samples respectively on the paperchromatogram. The R_f 0.77 compound was remained unidentified.

When R-acid, G-acid, N-W-acid and Schaeffer's acid were used instead of nitroso-R salt or the experiment was carried out in the N_2-atmosphere, the formation of gentisic acid from homogentisic acid was not demonstrated.

The spots on the paper were extracted with 10^{-4} M acetic acid buffer

TABLE II

Degradation of HGA by the Liver Extract of Rabbit in the Presence of Metalcatchers

Addition	Final concentration (μM)	O_2-uptake (μM)	Acetoacetic acid formed (μM)
None		1.90	0.90
Nitroso-R salt	10^{-3}	1.88	0.08
Oxine	10^{-3}	0.34	0.10
o-Phenanthroline	10^{-3}	0.21	0.00

HGA 2 μM, Time 90 minutes.

TABLE III

The Relation between Each Enzyme and Fraction

	Fraction	R_f 0.48	0.61	0.77	0.85	0.95
Ammonium sulfate	0.3 saturation	+	+	##	−	−
	0.3–0.5 saturation	+	+	#	+	+
	0.5–0.7 saturation	+	+	+	##	+
	0.7 saturation	−	−	−	−	−
0.5–0.7 saturation Ammonium sulfate	Adsorbable on Ca-gel	−	−	+	−	−
	Non-adsorbable on Ca-gel	+	+	−	+	+
Non-adsorbable fraction on Ca-gel	Isoelectric precipitation (pH 5.0)	+	+	−	−	−
	Non-precipitable (pH 5.0)	±	±	−	+	±

(pH 6.0) respectively and subjected to the enzymatic reaction (pH 7.2, 37.5° for 2 hours) and paperchromatography. Only one spot corresponding to GA was shown to appear, after incubation, with the diminution of other spots.

In every case of three metalcatchers, acetoacetic acid was not formed from HGA (Table II), even in the presence of nitroso-R salt, in which case the O_2-uptake was the same as in the control experiment.

It is quite logical that several enzymes should be required on the intermediate path way from HGA to GA, which was suggested in Table III.

These data will play a role for purification of each enzyme which is under investigation in this laboratory.

II. Formation of GA from GAL, DPGOA and DPGCA

Differential Determination of GA and GAL—Afterincubation, 2.0 ml. of 25 per cent H_2SO_4 and 2.0 ml. of 5 per cent phosphotungstic acid were added to the reaction mixture and filtered. The filtrate was extracted twice with two volumes of ether and the etherial layer was extracted with 5.0 ml. of *M*/2 phosphate buffer (pH 7.0). Only GA (318 mμ) was extracted into phos-

TABLE IV

Formation of GA

I. Urine

Compounds administered	GA excreted in urine (μg.)		R_f	
	24 hrs.	48 hrs.	Acetone : butanol : water	Benzene : acetate : water
GAL (30 mg.)	8290	250	0.95, 0.85	0.85, 0.49
DPGOA (30 mg.)	440	0	0.95, 0.85, 0.48	0.85, 0.49, 0.17
DPGCA (20 mg.)	13	0	0.95, 0.85, 0.60, 0.48	0.85, 0.49, 0.28, 0.17

II. Enzyme preparation

Substrate	Condition	R_f (Acetate : butanol : water)
GAL	Liver extract*	0.95, 0.85
DPGOA	Liver extract** or slice***	0.95, 0.85, 0.48
DPGCA	Liver extract** or slice***	0.95, 0.85, 0.61, 0.48

Notices: * Substrate 4 μM, enzyme 2.0 ml., 1 *M* acetate buffer (pH 5.5); 0.5 ml.; Total volume 5.0 ml., 90 min., 37.5°.

** Substrate 4 μM, enzyme 4.0 ml., 1 *M* phosphate buffer (pH 7.2) 1.5 ml., total volume 15.0 ml.; 3 hours, 37.5°.

*** Substrate 4 μM, slice 2.0 g., Ringer's solution 20.0 ml.; total volume 25.0 ml.; 3 hours, 37.5°.

phate layer and GAL (255 and 350 mμ) remained completely in the ether. The phosphate layer was used for fluorometrical determination of GA using H.G. 1 as the primary filter and B 470 as the secondary. 90.2 per cent of the added GA was recovered (*2*).

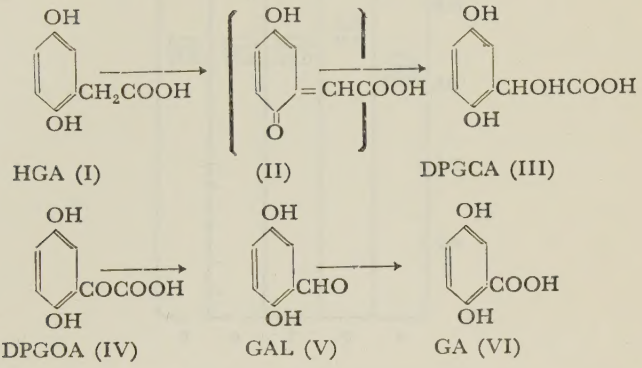
Results of the Experiments with the Normal Animal—30 mg. of compound was dissolved in a small amount of water and administered to a male guinea pig (weighing 250 g.) intraperitoneally and urine was collected for 24 hours in a bottle, to which 2 ml. of 10 per cent H_2SO_4 was added previously. GA in

the urine was determined as described in the previous paper (*2*) and the following results were obtained (Table IV).

From the above results, it is obvious that GA is formed from GAL; GAL and GA from DPGOA; DPGOA, GAL and GA from DPGCA.

DISCUSSION

These results seem to indicate that a series of the following reactions takes place in the formation of GA from HGA.



In the degradation of HGA by the liver extract of rabbit in the presence of added nitroso-R salt, five spots were demonstrated on the peperchromatogram. Among them four spots were identified with authentic samples III, IV, V and VI respectively and only R_f 0.77 was remained unidentied. When the R_f 0.77 compound was extracted with phosphate buffer and the enzymatic reaction was repeated with this compound, the spot of R_f 0.77 disappeared with the appearance of GA. Therefore the unidentified substance of R_f 0.77 is of much interest and may be a important intermediate.

Among metalcatchers tested, nitroso-R salt, oxine and *o*-phenanthroline were most effective on the enzyme actions. However, their effects were not uniform, e.g. in the case of nitroso-R salt the spot of R_f 0.85 compound was most marked and in that of oxine and *o*-phenanthroline the spots of R_fs. 0.48 and 0.61 were more remarkable (Table I). These facts seem to suggest, that each inhibitor may have the respective blocking point in the series of the reactions.

If the side chain of HPAA would be abbreviated by the liver extract and by the intact animal, SA and GA (*8*) would be formed, but both acids were detected neither in the urine of rabbit HPAA administered, nor in the reaction solution of liver enzyme (Fig. 1). Therefore the 2, 5-dihydroxy-group of HGA would be necessary for the abbreviation of its side chain.

SUMMARY

1. In the intact rabbit and its liver extract, in the presence of nitroso-R salt, the formation of GA from HGA, GAL, DPGCA and DPGOA was

demonstrated respectively. In the case of HGA four spots beside that of substrate were found on the paperchromatogram which were identified as GA, GAL, DPGCA and DPGOA. The R_f 0.77 compound was remained unidentified, though it could be metabolized to GA.

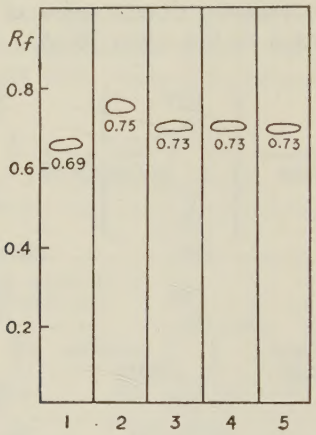


FIG. 1. Paperchromatogram of the reaction mixture of HPAA by liver extract.

(1) GA
(2) SA
(3) HPAA
(4) HPAA 60 μM, liver extract 20 ml.; pH 7.2, time 3 hrs.
(5) (4) plus nitroso-R salt: final concentration 10^{-3} M;
Solvent:
isopropanol: NH_4OH: water = 8 : 1 : 1
Notice: The spots corresponding to GA, SA and HPAA respectively are easily distinguished by their behavior to the ultraviolet-light. GA shows bluish-white fluorescence, SA greenish-white to the ultraviolet-light, while HPAA absorbs the ultraviolet-light.

2. Among 10 metalcatchers tested, nitroso-R salt, oxine and *o*-phenanthroline were effective on the formation of GA, and seem to block the different point of this pathway individually. R-acid, G-acid, Schaeffer's acid and N-W-acid were proved to be inert.

3. Since the side chain of HPAA was not abbreviated to form SA, 2, 5-dihydroxy group of HGA is essential for the abbreviation of its acetate rest in the organism.

The authors are indebted to Dr. T. Sakan for synthesizing of GAL, DPGCA and DPGOA.

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TRANS-CINNAMIC ACID AMIDE AS A METABOLIC PRODUCT OF STREPTOMYCES

By YASUHARU SEKIZAWA

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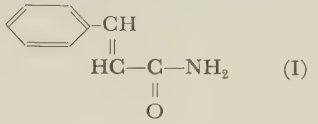
(Received for publication, July 26, 1957)

During the continuing researches on the metabolites of Genus *Streptomyces*, it was recognized that trans-cinnamic acid amide (I) was accumulated in the cultured broth of an unidentified *Streptomyces* No. 902 under proper cultural condition.* Trans-cinnamic acid amide (I) has no characteristic cinnamic odor, but the accumulation of this metabolite seems to be noted relating to the various characteristic odors of the cultures of *Streptomyces*.



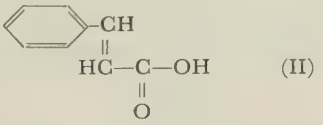
(I)

This metabolite was at first obtained in the prismal crystalline form from the concentrate of the *n*-butylacetate extract of the cultured broth filtrate. After several times recrystallization from acetone-ethylacetate system (8: 1 *v/v*), it melts at 147–149°, and is optically inactive in methanol. The ultraviolet absorption spectrum in water showes maxima at 275 mμ ($E^{1\%}_{1\,cm}$ = 1330) and 216 mμ ($E^{1\%}_{1\,cm}$ = 1093), and explains the presence of phenyl group and especially an double bond conjugated with carbonyl group.

The analytical data of the sample dried at 60°, 8 hours under 10^{-1}mm. Hg on P_2O_5 show C, 73.61 per cent; H, 6.61 per cent; N, 9.24 per cent and no presence of halogen or sulfur. The molecular weight by Rast method with camphor matches to 149. Calculated for C_9H_9NO, C, 73,43 per cent; H, 6.17 per cent; N, 9.52 per cent and theoretical for molecular weight, 147. 1.

Bayer's double bond test is positive. Ninhydrin, biuret, Ehrlich's aldehyde and nitroprusside tests are negative.

This metabolite is easily converted to trans-cinnamic acid (II) when



(II)

* The culture of *Streptomyces* No. 902 was isolated by Mr. M. Arishima from a soil in our laboratory. The cultural condition will be described in the following paper related to this experiment.

treated with boiling conc. hydrochloric acid under reflex condenser.

On the other hand, when this metabolite is steam-distilled under drastic alkaline condition, 1 mole of the metabolite gives rise to 1 mole of ammonium.

So the metabolite was identified as the amide of trans-cinnamic acid (*I*) having the structural formula (I).

EXPERIMENTAL AND RESULTS

The Conversion of C_9H_9NO to Trans-cinnamic Acid

Four hundred milligrams of the metabolite, C_9H_9NO, is boiled with 10 ml. of conc. hydrochloric acid on asbestos net for 4 hours under reflex condenser. After the dissolution of the crystal, flaky crystalline substance was formed. Yield, 350 mg. The recrystallized substance from ethylacetate melts at 132–134°. The analytical data of the sample dried at 60°, 8 hours under 10^{-1} mm. Hg on P_2O_5 show C, 72.78 per cent; H, 5.68 per cent and no presence of nitrogen. The molecular weight by titration matches to 150 as monobasic acid. Calculated for $C_9H_8O_2$, C, 72.94 per cent; H, 5.45 per

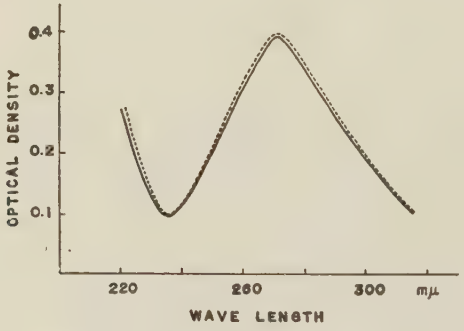


Fig. 1. The ultraviolet absorption spectrum of the degradation product $C_9H_8O_2$ comparing with trans-cinnamic acid.
····· trans-cinnamic acid, —— degradation product $C_9H_8O_3$

cent and theoretical for molecular weight, 148.1. This degradation product has the characteristic cinnamic odor. And the ultraviolet absorption spectrum in 10^{-1} *N* NaOH quite agrees with the authentic trans-cinnamic acid as shown in Fig. 1; natural cinnamic acid 270 mμ ($E^{1\%}_{1\,cm}=1184$); authentic trans-cinnamic acid 270 mμ ($E^{1\%}_{1\,cm}=1189$). The mixed melting point test also showes these two substances are identical and that the form of this naturally occuring cinnamic acid in geometrical isomerism is trans.

SUMMARY

The amide of trans-cinnamic acid (I) was recognized as the metabolite of an unidentified *Streptomyces* No. 902.

Author gratefully thanks to Prof. Dr. Y. Nakamura for his many kind advices and

Mrs. Y. Baba, Tokyo Pharmaceutical University, for micro elementary analyses. Author was also indebted to Mr. M. Arishima for his advices in microbiological field, and to Mr. M. Ogasawara for the pilot plant fermentation and to Mrs. K. Harada for the skilful assistance.

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PREPARATION AND CHROMATOGRAPHY OF THE HYDROXAMIC ACIDS OF SOME BILE ACIDS

By KYUTARO SHIMIZU, TOSHIAKI USUI, TADASHI FUJIOKA and KAZUMI YAMASAKI

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(Received for publication, July 30, 1957)

In order to odtain a chromatogram of the bile acids and furthermore, to determine each of them colorimetrically, it is desirable to prepare such derivatives as are *per se*, or readily convertible into, colored substances.

Silberman and Silberman-Martyncewa (*1*) have made an attempt to prepare a colored derivative of some bile acids to get a chromatogram thereof. Later Tanaka and Takeda (*2*) reported that the Girard T hydrazones of some keto bile acids were separable by paper chromatography, eaeh spot of the hydrazones being detected by some coloring reagent.

Guddu *et al.* (*3*) recently showed that fatty acids could be determined colorimetrically after conversion into their hydraxamic acids, which in the presence of ferric ions turned violet in color.

In the present report we describe the preparation and chromatography of the hydroxamic acids of some bile acids, which were proved to behave and be colorimetrically determinable, just like those of fatty acids.

EXPERIMENTAL

I. Preparation of the Hydroxamic Acids of Some Bile Acids

Cholyl Hydroxamic Acid—To a methanolic solution of methyl cholate (2g.; m.p. 155°) was added a mixture of equal volume (25 ml.) of 12.5 per cent methanolic KOH solution and 12.5 per cent methanolic hydroxylamine solution, and refluxed for 5 minutes.

After being cooled, the mixture was diluted with an equal quantity of water, acidified (lithmus) by adding acetic acid, and evaporated on a water bath until no bubbling was observed, when a crystalline substance began to separate.

After standing overnight at room temperature, the crystales were collected. (Yield 1.9g.) Recrystallizations from aqueous methanol afforded a pure sample (needles) of cholyl hydroxamic acid, melting at 108–109°; $E_{216}^{alc.}=65.7$

Analysis. Calcd. for $C_{24}H_{41}O_5N$ (423.58) : N, 3.31
Found : N, 3.27

The hydroxamic acid derivatives of some other bile acids were prepared just like the cholyl hydroxamic acid, and some properties of them are

summarized in Table I.

Absorption Spectra of Ferric Hydroxamates of Some Bile Acids The ferric hydroxamate was prepared as follows: To an aliquot of methanolic solution of the hydroxamic acid was added a drop of 0.5 per cent ferric perchlorate solution and the solution was extracted with butanol. The butanol layer was washed with water, the absorption spectrum of which was observed.

The spectra of some hydroxamates so obtained are presented in Fig. 1.

TABLE I

Hydroxamic Acids of Some Bile Acids

Hydroxamic acid	Crystal form	m.p.	Per cent of nitrogen*
Cholyl	needles (methanol)	109°	3.27 (3.31)
Deoxycholyl	tiny needles (methanol)	181°	3.59 (3.34)
Chenodeoxycholyl	amorphous powder	——	——
Hyodeoxycholyl	Prisms (methanol)	154°	3.33 (3.34)
Lithocholyl	needles (methanol)	187°	3.56 (3.58)
Δ^5-3β-Hydroxycholenyl	needles (aqueous methanol)	107°	3.72 (3.60)

* Each figure in parentheses indicates the calculated N value of the corresponding hydroxamic acid.

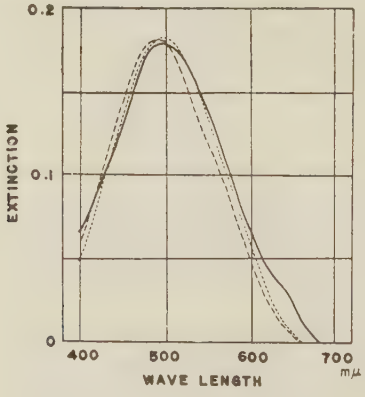

FIG. 1. Absorption spectra of ferric hydroxamates of cholic (————), deoxycholic (·····) and lithocholic (— — —) acids.

As shown in Fig. 1, the molar absorption maximum of every sample here studied lies at the same region of wave length (500 mμ) and is of a nearly equal intensity.

II. Paper Chromatography of the Hydroxamic Acids

The hydroxamic acids described above underwent paper chromotography ('Toyo' filter paper No. 50; ascending method) as usual, the solvent

system being as follows: toluene/acetic acid/water (10:10:2). The spot on the paper was detected by spraying 1 per cent solution of ferric chloride in isopropyl ether. The chromatogram and R_f values of these acids are shown in Fig. 2 and in Table II.

TABLE II

R_f Values of the Hydroxamic Acids of Some Bile Acids

Hydroxamic acid	R_f
Lithocholyl	0.57
Deoxycholyl	0.34
Chenodeoxycholyl	0.22
Hydeoxycholyl	0.09
Cholyl	0.06

Paper: 'Toyo' filter paper No. 50; solvent: toluene/acetic acid/water (10:10:2); ascending method, *ca.* 2 hours, 20 cm.; spot d tection: 1 per cent $FeCl_3$ in isopropyl ether.

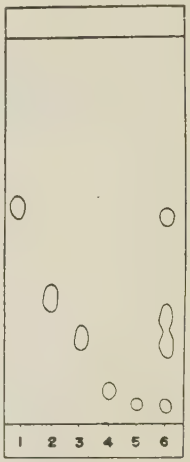

FIG. 2. Paper chromatogram of hydroxamic acid derivatives of some bile acids.

Solvent: toluene/acetic acid/water (50: 50: 10)

1. Lithocholyl hydroxamic acid
2. Deoxycholyl hydroxamic acid
3. Chenodeoxycholyl hydroxamic acid
4. Hyodeoxycholyl hydroxamic acid
5. Cholyl hydroxamic acid
6. A mixture of lithocholyl, deoxycholyl, chenodeoxycholyl and cholyl hydroxamic acids

III Column Chromatography of the Hydroxamic Acids

Solvent:

1. 50 per cent acetic acid, saturated with petroleum ether,
2. petroleum ether (b.p. 40–60°),
3. petroleum ether/isopropyl ether (60:40),
4. petroleum ether/isopropyl ether (40:60),
5. petroleum ether/isopropyl ether (20:80),
6. isopropyl ether.

These solvent mixtures (Solvent 2–6) were saturated with 50 per cent acetic acid.

Ferric Perchlorate Reagent—Dissolve 0.8 g. of iron wire in 10 ml. of 60 per cent perchloric acid, transfer the solution to a 100-ml. volumetric flask with 10 ml. of water and then add methanol up to the mark to obtain the stock solution.

To prepare the reagent solution, transfer 40 ml. of the stock solution and 18 ml. of 60 per cent perchloric acid to a 1-liter volumetric flask and then add methanol up to the mark.

The *celite column* is prepared as follows: Six g. of celite (dried at 130°) is mixed well with 5 ml. of 50 per cent acetic acid (Solvent 1), suspended in petroleum ether (Solvent 2) and poured into a glass tube (1.3 cm.×30 cm.).

Each sample of the hydroxamic acids, above mentioned, was dissolved in 0.4 ml. of 50 per cent acetic acid, mixed well with 0.5 g. of celite, suspended in petroleum ether and poured onto the column of celite described adove.

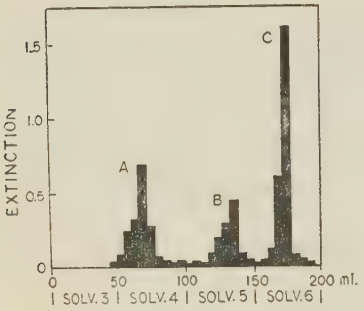

Fig. 3. Column chromatography of hydroxamic acids of some bile acids.
A : lithocholyl hydroxamic acid (16.5 μM),
B : deoxycholyl hydroxamic acid (13.3 μM),
C : cholyl hydroxamic acid (16.3 μM)

Fifty ml. of each of the above solvents (Solvent 2–6) was poured successively onto the column of celite and each 5 ml. of eluate was collected (fraction collector), evaporated and 10ml. of ferric perchlorate reagent was added.

Afrer standing for 10 minutes the transmittance of the mixture was read photometrically against the blank (ferric perchlorate reagent), using the 530mμ filter.

The results are presented in Fig. 3. As shown in Fig. 3, lithocholyl hydroxamic acid was eluted by Solvent 4, deoxycholyl and chenodeoxycholyl hydroxamic acids by Solvent 5 and cholyl hydroxamic acid by Solvent 6.

SUMMARY

Hydroxamic acids of some bile acids were prepared and paper as well as column chromatography of these acids were presented, where those of deoxycholic and chenodeoxycholic acids were shown to be separable by the paper chromatography.

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THE EFFECT OF METAL IONS ON ALKALINE PHOSPHATASE

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(Received for publication, July, 31, 1957)

Alkaline phosphomonoesterase was assumed to be a magnesium-apoenzyme complex (*1*). The activity observable without any extra addition of magnesium ion was attributed to the presence of trace of this ion in the test mixture (*1*). Besides magnesium, some kind of heavy metals seems to have been contained in the enzyme solution hitherto used, and activation by amino acids and their related compounds (*2*, *3*) was explained by deprivation of inhibitory effect of those heavy metal ions through chelate formation (*1*, *4*).

The present paper reveals that the combination of zinc ion, one of inhibiting metal ions, is competed by magnesium ion, whereas copper ion combines firmly with apoenzyme, causing non-competitive inhibition of magnesium-activated monoesterase. The amount of magnesium and zinc contained in the dialyzed swine kidney autolysate, when quantitatively analysed, agreed fairly well with the values judged as pre-existant in the enzyme solution through analytical treatment of the activity curve obtained in experiments with extra addition of those metal ions.

EXPERIMENTAL AND RESULT

Enzyme Solution—Swine kidney homogenate was autolyzed with five volumes of water and a small amount of chloroform and toluene, at 37° for 3 days, and the supernatant fluid was dialyzed for 2 days against running water, for another 2 days against water desalted by ion exchange resins and stored in an ice box.

Analysis of Zinc, Magnesium, and Manganese in the Dialyzed Autolysate—80 ml. of the dialyzed autolysate was evaporated in a platinum crucible, ignited after addition of 10 ml. of concentrated nitric acid, and dissolved in 4 ml. of 0.1 *N* sulfuric acid. 2 ml. of this ash solution was neutralized to pH 4 with 0.1 *N* sodium hydroxide, and made up to 5 ml. by addition of 1 *N* potassium chloride used as supporting electrolyte for polarography, an apparatus manufactured by Yanagimoto Co. being used. A rapid increase of current was noticed at a position of half-wave potential indicating the presence of zinc, and its height was 2.3 cm. (0.92 μ amp.). Since the height of the wave of 10^{-4} *M* zinc solution containing potassium chloride was 0.75 cm. (0.30 μ amp.), the concentration of zinc in the diluted ash solution used for polarography was 3×10^{-4} *M*, and accordingly that in the dialyzed autolysate

would be $3\times10^{-4}\,M\times0.05\times2.5=3.8\times10^{-5}\,M$. In the polarogram the presence of manganese was difficult to detect, as its possible position of current increase was overlapped by the reduction wave of hydrogen ion. No existence of heavy metals other than zinc was indicated in the range of applied electromotive forces increasing from zero to—1.5 volts. Analysis of veronal sodium used for the preparation of buffer solution was carried out in a similar manner; 6.18 g. of this reagent was ignited, the ash was disolved in a small amount of water, neutralized with 1 N sulfuric acid, evaporated to dryness, and dissolved in 6 ml. of 0.1 N sulfuric acid. 2ml. of this ash solution was neutralized with 0.1 N sodium hydroxide and diluted to 5 ml. with water. In a similar polarography neither zinc nor other heavy metals was detected.

Then the estimation of magnesium was carried out by Yamada's colorimetric method (*5*). The solution containing supporting electrolyse used above for polarograpy was diluted 100 times with water and to 1 ml. of it was added 1 ml. of the saturated solution of tropeolin 00. The precipitate was washed with a mixture of acetone and ether (1 : 1), dissolved in 10 per cent sulfuric acid, and the pink color developed was colorimetrically estimated against the standard solution similarly prepared from $10^{-4}\,M$ magnesium sulfate solution. The concentration of magnesium in the sample thus determined was $0.38\times10^{-4}\,M$, therefore, that in the dialyzed autolysate would be $0.38\times10^{-4}\,M\times0.05\times2.5\times100=4.8\times10^{-4}\,M$, while no magnesium was detected in the ash solution from the buffer reagent.

For colorimetric estimation of manganese in the dialyzed autolysate the method of Dubuisson (*6*) was applied. To another one ml. of the ash solution in 0.1 N sulfuric acid, one drop of 0.5 per cent silver nitrate, and 8 drops of 1 per cent potassium persulfate, and the mixture was warmed at 50° for 5 minutes. The concentration of permanganate produced was $3.3\times10^{-5}\,M$, therefore, that in the dialysed autolysate was $3.3\times10^{-5}\,M\times0.05=1.6\times10^{-6}\,M$. No manganese was detected in the ash solution prepared from veronal sodium mentioned above.

Enzymatic Hydrolysis of Disodium p-Nitrophenylphosphate—A mixture of 1 ml. of diluted autolysate, 1 ml. 0.1 M veronal sodium-HCl (pH 9), and 2 ml. water or solutions of substances to be tested was preincubated at 37° for 10 minutes, and than 1 ml. of similarly warmed $2.5\times10^{-3}\,M$ p-nitrophenylphosphate solution was added. The dialyzed autolysate was previously diluted with 19 volumes of water so that 15 per cent hydrolysis took place in 1 hour when 2 ml. of $2.5\times10^{-3}\,M$ magnesium chloride was added for maximum activation. The ash analysis of the dialyzed autolysate, indicates the concentrations of zinc, magnesium and manganese in the test solution would be $3.8\times10^{-7}\,M$, $4.8\times10^{-6}\,M$, and $1.6\times10^{-8}\,M$, respectively. After incubation for 30 and 60 minutes at 37°, 2 ml. aliquot of the test solution was introduced into equal amount of saturated sodium carbonate solution. Deproteinization was omitted as the test solutions were colorless and showed no turbidity by trichloroacetic acid addition. p-Nitrophenol liberated was estimated by electrospectrophotometry ($\lambda=400\,m\mu$). Since the degree of

hydrolysis increased in direct proportion to time up to 60 minutes, the relative activity is expressed as the ratio of extinction coefficients observed at the 60-minute test to that in the case of maximum exhibited by single addition of 10^{-3} *M* Mg. In the following discription the symbols p[Mg], p[Ligand], *etc.* are used to denote negative logarithms of the concentrations of respective metal ions or chelating substances, as usally used for hydrogen ion concentration.

Activity With or Without the Addition of Magnesium—As described above, the dialyzed autolysate itself was slightly active even without the addition of magnesium, and its activity was about 20 per cent of the maximum activity

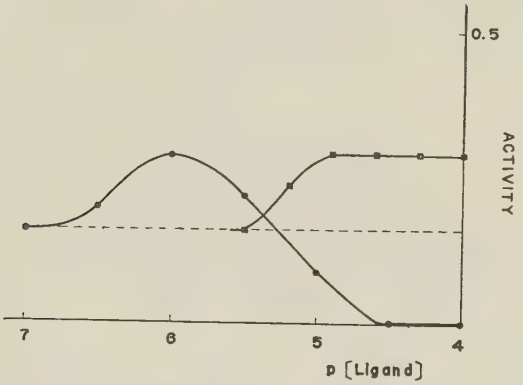

FIG. 1. Effect of ethylendiamine (ED) and ethylenediamine tetraace tate (EDTA) on alkaline phosphomonoesterase.

Relative activities are expressed by taking the maximum activity observed in the presence of 10^{-3} *M* Mg as 1.0; p[Ligand] is the negative logarithm of concentrations of ligands in the reaction mixture. One ml. of enzyme (diluted dialysate of autolyzed swine kidney), 1 ml. of 0.1 *M* veronal buffer (pH 9.0), 1 ml. of ED or EDTA solution, and 1 ml. of water were mixed and preincubated at 37° for 10 minutes. One ml. of 2.5×10^{-3}*M* *p*-nitrophenyl-phosphate solution similarly warmed was added. After 60 minutes, the amount of *p*-nitrophenol liberated was photometrically estimated. The control test solution for maximum activation contained 1 ml. of 5.0×10^{-3}*M* $MgCl_2$ instead of the ligand. ■ ED; ● EDTA.

produced by the addition of 10^{-3} *M* magnesium. However, this maximum is not heightened by extra addition of histidine or ED. On the other hand, when extra magnesium addition was omitted, ethylenediamine (ED) resulted in a slight increase of activity. Ethylenediamine tetraacetate (EDTA) behaved similarly as ED, but when its concentration is increased, the enzymic activity becomes lower (Fig. 1).

The fact that the slight activating effect of ED, EDTA, or amino acids was detected in the case of no extra addition of Mg, and that the maximum activity observable with addition of 10^{-3} *M* magnesium was no more heightened by the presence of those chelating agents, indicates that enzyme partially

exists in the dialysed autolysate as an inactive complex with some inhibitory metals, which could be deprived from the complex by magnesium or by chelating compounds.

Activation in the Presence of ED by Magnesium of Various Concentrations—The p[Mg]-activity curve in the presence of ED of $1.2\times10^{-5}M$ concentration was obtained by plotting tne relative activity against various p[Mg] (dotted line of A in Fig. 2). In the absence of ED, a similar p[Mg]-activity curve was obtained (dotted line of B in Fig. 2). However, the concentrations of magnesium required for the same extent of activation are lower in the present of ED than in its absence, though the same maximum activity is finally achieved. Under an assumption that the enzyme protein could become active when reversibly combined with magnesium, the concentration of magnesium, which

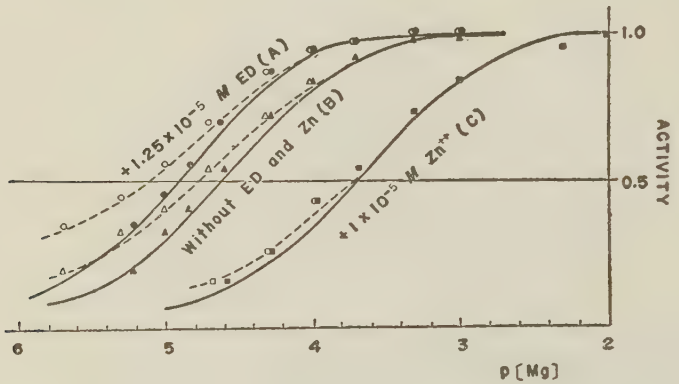

Fig. 2. p[Mg]-Activity curve in the presence of ED or Zn.
Same condition as in Fig. 1, except that 1ml. of $6.25\times10^{-5}M$ ED or $5\times10^{-5}M$ $ZnCl_2$, was used instead of the ligand and 1 ml. of various concentrations of $MgCl_2$, instead of water. △ without ED and Zn, ○ ED, □ Zn. The symbols ▲, ●, ■ (solid curves) indicate, at the corresponding ones △, ○, □ (dotted curves), the activities by the total Mg concentrations corrected respectively with regard to the Mg concentration ($5.0\times10^{-6}M$ obtained graphically at Fig. 3) contained a priori in the reaction mixture.

would be contained a priori in the test solution, was calculated by graphical analisis (Fig. 3) of the dotted curve of A in Fig. 2, according to the following equation, where K_{Mg} is a dissociation constant of enzyme protein-magnesium complex, and x and [Mg] are respectively the concentration of Mg contained a priori in the enzyme solution and that extra added.

$$\frac{V}{V_{max}}=\frac{([Mg]+x)}{([Mg]+x)+K_{Mg}} \quad \cdots\cdots(1)$$

$$([Mg]+x)(V_{max}-V)=V\times K_{Mg}$$

$$[Mg]=\frac{V}{V_{max}-V}K_{Mg}-x \quad \cdots\cdots(2)$$

The value x was $0.5\times10^{-5}M$, which agreed fairly well with the result

of colorimetric analysis mentioned above. Regarding the sum of this amount and those of extra-added of magnesium as the actual concentrations of magnesium in the test solutions, p[Mg]-activity curve was drawn (solid curve of A in Fig. 2). It was a sigmoid of the first order, indicating that pK_{Mg} is 4.9. The p[Mg]-activity curve in the absence of ED, when similarly corrected, ran parallel to that obtained above, simply being located by 0.3 p[Mg] unit towards the activity axis (solid curve of B in Fig. 2).

pMg Activity Curve in the Case of Zinc Addition of 10^{-5} *M Concentration*— Fig. 2 shows that the concentration of Mg to be added for producing the same

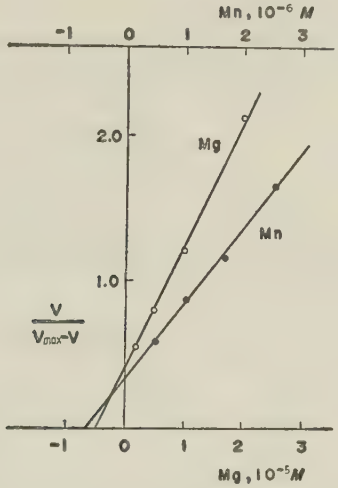

Fig. 3. Calculation of magnesium and manganese concentration presented in reaction mixture.

Ordinate, $\frac{V}{V_{max}-V}$ (V=relative activities by various concentrations of Mg or Mn in the presence of ED, V_{max}=the maximum activity expressed by 1.0 in the presence of 10^{-3} *M* Mg); Abcissa, Mg or Mn=the final concentration of Mg or Mn added in reaction mixture. These lines are obtained by plotting the data indicating the activation by Mg in Fig. 2 or by Mn in Fig. 5 in the presence of ED.

activity was higher in this case than when zinc ion was not added, and still much higer than that when zinc ion primarily contained was chelated by ED. When p[Mg]-activity curve is drawn, taking this amount of Mg in consideration, a sigmoid curve C of the first order is obtained, the dislocation of pK_{Mg} from that of curve A being 1.2 p[Mg] unit, where pK_{Mg} is the negative logarithm of the dissociation constant of apoenzyme-magnesium complex. A competition between magnesium and zinc for enzyme protein was thus ascertained.

According to the theory of Michaelis and Menten, the extent of dislocation (−1.2) of pK_{Mg} due to zinc added as a competitive inhibitor should

be $\log K_{Zn} - \log(Zn + K_{Zn})$ where K_{Zn} is the dissociation constant of apoenzyme-zinc complex. The concentration of zinc was 10^{-5} mole, if judging from the result of polarographic analysis, the amount of zinc contained a priori in the test solution was neglected. K_{Zn} thus obtained was 6.6×10^{-7}. This K_{Zn} value was then used for calculating the zinc concentration y contained primarily in the test solution by solving the equation $[-\log(6.6\times10^{-7}) + \log([y] + 6.6\times10^{-7}) = 0.3]$. The value of y obtained was $6.6\times10^{-7}\,M$, nearly the same as that of $3.8\times10^{-7}\,M$ measured by polarography.

Inhibition by Copper and Activation by Manganese—Copper is a strong and noncompetitive inhibitor. (see Fig. 4)

On the other hand, manganese and cobalt exhibit activating effect, similar to Mg. Estimation of p[Co]-activity curve was omitted since no cobalt ion was detected in the autolysate. p[Mn]-Activity curve obtained in the

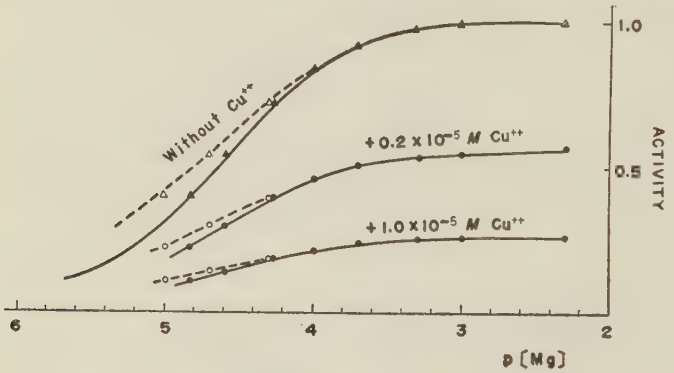

Fig. 4. Activity curve in the presence of 0.2 or $1.0\times10^{-5}M$ Cu. Same conditions as in Fig. 2, except that 1 ml. of $CuCl_2$ solution was used in stead of $ZnCl_2$. ▲, without Cu, and the activity is corrected by total Mg as in the case of Fig. 2; O, activity obtained by the addition of Mg; ●, activity corrected by total Mg.

presence of $1.2\times10^{-5}M$ ED, a chelate compound of which with manganese has a far smaller stability constant than that with zinc, was a bell shape, composed of an ascending and descending portions and the top of the curve remained below and at nearly 80 per cent of the maximum attainable by magnesium activation. It was noticed that the ascending portion indicated some deviation from a sigmoid curve of the first order. Therefore, an assumption was made that a minute amount of manganese would be present a priori in the dialysed autolysate and it might have caused a light activation. As in the case of magnesium ion, graphic method was undertaken to calculate that manganese concentration, which might have been present in the test solution. Fig. 3 indicates that this value should be $6.5\times10^{-7}M$. It is remarkable this concentration is far higher than the value anticipated from the result of colorimetric determinaion mentioned above. As a trial, when p[Mn]-activity curve was corrected by that value of $6.5\times10^{-7}\,M$, a typical

ascending portion of sigmoid nature was obtained (Fig. 5), indicating pK_{Mn} would be 5.73. The descending part of the curve must be ascribed to combination of the active apoenzyme-manganese complex with second manganese ion to form an inactive complex.

Now, the discrepancy between colorimetrically and enzymatically analyzed concentrations of manganese was clarified as follows: When E_o is the concentration of a free enzyme, E_{Mg} that of active enzyme combined with magnesium, and E_{Mn} that combined with manganese, the total concentration of the enzyme, E_t, and the dissociation constants K_{Mg} and K_{Mn} of respective forms of the active enzyme will be represented as follows:

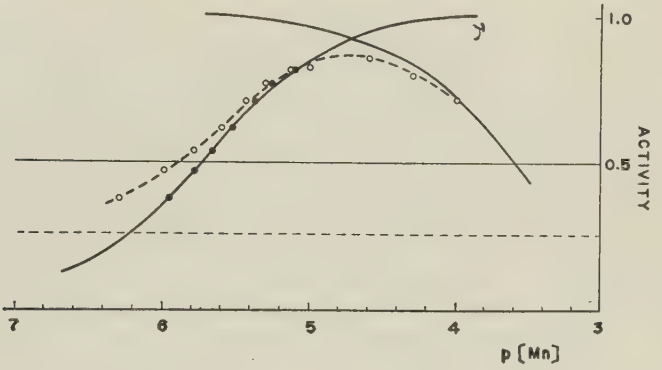

FIG. 5. p[Mn[-Activity curve in the presence of ED.

Same conditions as in Fig. 2, except that 1 ml. of various concentrations of $MnSO_4$ solution was used instead of Mg. ○, activity obtained by the addition of Mg; ●, activity corrected by total Mg.

$$E_t = E_o + E_{Mg} + E_{Mn}$$

$$\frac{(E_o)\,(Mg)}{E_{Mg}} = K_{Mg}, \qquad \frac{(E_o)\,(Mn)}{E_{Mn}} = K_{Mn}$$

Then,

$$E_t = E_o + \frac{(E_o)\,(Mg)}{K_{Mg}} + \frac{(E_o)\,(Mn)}{K_{Mn}} \qquad \text{.....(4)}$$

$$\frac{E_t}{E_o} = 1 + \frac{(Mg)}{K_{Mg}} + \frac{(Mn)}{K_{Mn}}$$

$$\text{Activity} = \frac{E_t - E_o}{E_t} = \frac{\dfrac{(Mg)}{K_{Mg}} + \dfrac{(Mn)}{K_{Mn}}}{1 + \dfrac{(Mg)}{K_{Mg}} + \dfrac{(Mn)}{K_{Mn}}} \qquad \text{.....(5)}$$

If either (Mg) or (Mn) is 0, the equation 5 would be reduced to the following epuation 6, which is no other than those applied for calculation of magnesium or manganese concentration, assumed to be present in the test solution and revealed that they were $4.80 \times 10^{-6} M$ for magnesium and $6.5 \times 10^{-7} M$ for manganese. The dissociation constants K_{Mn} and K_{Mg} were 1.85×10^{-6}

and 1.25×10^{-5}, respectively, as mentioned above.

$$\text{Activity}=\frac{(\text{Mg})}{K_{\text{Mg}}+(\text{Mg})}=\frac{(\text{Mn})}{K_{\text{Mn}}+(\text{Mn})} \quad \text{.....(6)}$$

It is remarkable that both terms of numerator in the equation 5 are nearly equal. This means that the metal which would have been present in the dialyzed monoesterase solution could be manganese as well as magnesium. However, the activating metal present in the enzyme solution must be magnesium since the colorimetrically estimated magnesium concentration was $4.8\times10^{-6}M$, while manganese measured by colorimetry was $0.16\times10^{-7}M$ and it would be too low to exhibit any activation of apoenzyme, as seen from p[Mn]-activity curve (Fig. 5).

DISCUSSION

The experiments presented in the paper indicate that the alkaline phosphomonoesterase of swine kidney is transformed to an active enzyme by combination with magnesium. This ion can be replaced by manganese ion. However, the activating agent in physiological means would be magnesium ion from consideration of the concentration present in body fluid. An inhibitory effect of zinc ion is caused by the formation of an enzyme-zinc com plex, from which this ion, however, can be competitively expelled by magnesium ion. The inhibition by zinc ion can also be removed by some chelating agents such as ethylenediamine or ethylenediamine tetraacetate. However, an excess of EDTA inhibits the monoesterase action, and the mechanism of this inhibition will be discussed elsewhere (*7*), together with the action of some other chelating compounds.

Mathies (*8*) reported that among the heavy metals such as zinc, magnesium, copper, iron, *etc.*, detected in a purified kidney phosphatase, copper was of inhibitors and found its way into the enzyme preparation during the purification process. The present experiment has shown that copper noncompetitively inhibits the magnesium-activated monoesterase and that the hydrolysis of *p*-nitrophenyl phosphate even in the absence of ED could attain the maximum rate by the single addition of magnesium, and this result speaks rather against the existence of copper in the enzyme solution. Iron ion was present in about $3.3\times10^{-5}M$ in the enzyme solution, when estimated by potassium ferrocyanide method, hence would be in $0.33\times10^{-6}M$ in the test solution. Iron of such a low concentration was found not to interfere with the activity of the enzyme. Calcium ion detected in the ash solution prepared from the enzyme solution was also too minute to show any effect on the hydrolysis. There are several reports on the activation of monoesterase by heavy metals (*9–13*). However, so far as investigated here, it was impossible to find the presence in effective amount of any activating metals other than magnesium in the autolysate.

SUMMARY

The alkaline phosphomonoesterase is a magnesium-apoenzyme complex.

In the dialyzed swine kidney autolysate, magnesium, zinc, manganese, calcium and iron were found but no copper.

A slight hydrolytic action, even without the addition of magnesium, is due to this ion present in the autolysate itself. Its concentration was calculated to be $4.8\times10^{-6}M$ in the hydrolyzing test solution through analysis of the activity curve and the value agreed approximately with that obtained by colorimetric analysis of the dialyzed autolysate.

Zinc ion is inhibitory to apoenzyme by competition with magnesium. Some chelating reagents, for instance, ethylenediamine capable of complex formation with zinc could slightly activate the enzyme of the dialyzed autolysate.

Polarographic estimation of zinc concentration in the autolysate revealed it to be $3.8\times10^{-7}M$. This value nearly agreed with $6.9\times10^{-7}M$, calculated from the point of competition with magnesium.

Manganese salt was effective for activation of enzyme at lower concentrations than magnesium. However, at its higher concentration an inhibition took place because of formation of inactive complex with the second manganese ion. The result of manganese analysis in the autolysate showed that the concentration of manganese in the test solution was so low that any activation of the enzyme could not take place.

The inhibition by copper was non-competitive with magnesium.

The author wishes to express his sincere thanks to Prof. S. Akamatsu for his kind guidance in this work. This investigation was supported by a Grant in Aid for Scientific Research from the Ministry of Education to Prof. S. Akamatsu.

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The Journal of Biochemistry, Vol. 45, No. 1, 1958

STUDIES ON PROTAMINES

II. DETERMINATION OF THE END GROUPS OF CLUPEINE AND SALMINE AND THEIR MOLECULAR WEIGHT*

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(Received for publication, August 7, 1957)

In the previous paper of this series, some informations on the separation and purification, qualitative amino acid composition and inhomogeneity of clupeine and salmine have been reported (*1*). The present paper deals with the molecular weight estimation from the results of the end group determination of these protamines.

It has been reported that the molecular weight of both clupeine and salmine is of the order of several thousands. To such a protein of lower molecular weight and of simpler structure, an application of molecular weight determination procedure by the end group assay seems to be of particular significance. As described in the previous paper (*1*), the main fraction of clupeine and salmine consists only of arginine and few kinds of monoamino-monocarboxylic acid, and contains none of the other basic, acidic and sulfur-containing amino acids. Hence it is very probable to assume, that each of these protamines is composed of a single polypeptide chain. In fact, Rasmussen *et al.* (*2*) have proved the validity of this assumption by an electrometric titration of clupeine and calculated its molecular weight. Felix *et al.* (*3*, *4*) have tried to estimate the molecular weight of clupeine from the result of the end carboxyl group determination of the methyl ester, prepared using methanol saturated with hydrogen chloride, and also from the result of formol titration of the N-end group. However, Šorm and coworkers (*5*, *6*) have followed the above experiment and found that the methoxyl content in the clupeine ester showed a value which was not expected from the molecular weight based on the diffusion-viscosity method.

Fraenkel-Conrat *et al.* (*7*) have reported that an esterification of salmine could hardly be observed by their mild method (*8*) of esterification which is carried out in methanol containing hydrochloric acid, and is specific for the carboxyl groups of proteins. They also have indicated that no detectable amount of amino or imino group could be found in salmine by methods of ninhydrin, nitrous acid and formol titration (*7*). From these results they supposed that salmine might be a cyclic polypeptide. This

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result, however, was in contrast with the findings of English researchers on their salmine, that the N-terminal residue was identified as proline by DNP-method first applied to the protamine by Porter *et al.* (*9*) and could be determined quantitatively by formol titration carried out by Tristram (*10*).

In the last several years, identification and determination of N-terminal residues of clupeine and salmine have been studied in our laboratory by various methods such as methyl esterification, dinitrophenylation, nitrous acid, and electrometric titration. Parts of these results obtained have already been briefly reported (*11*, *12*) and the identification of N-terminal residues on the basis of ultraviolet absorption measurements on DNP-protamines and molecular weight estimation as a result have also been demonstrated in detail (*13*). In the present communication, determination of terminal carboxyl groups of clupeine and salmine by methyl esterification methods according to Fraenkel-Conrat and Felix, determination of N-terminal groups by manometric Van Slyke method, and estimation of their molecular weight based on these results are discussed in detail.

EXPERIMENTAL

I. Preparation of Clupeine and Salmine Methylesters

A. Materials—The less and the more soluble clupeine sulfate (1947) and the less soluble salmine sulfate (1950) preparations were derived from the corresponding picrate fractions of the protamines, respectively, and purified as described in the previous paper (*1*). Crude unfractionated picrate preparations of clupeine (April 3, 1951) and salmine (1950) were also used for the esterification. The date in parentheses represents that on which the fish used as the source of the protamine was obtained (*1*).

B. Esterification with Methanol Containing 0.1N HCl—(*a*) According to the method by Fraenkel-Conrat and Olcott (*8*), the less soluble clupeine sulfate (500mg.) was suspended in anhydrous methanol (50ml.), and to this concentrated HCl (*ca.* 11 *N*, 0.5 ml.) was added with cooling. After the mixture was left to stand at room temperature with occational shaking for 43.5 hours (during the period, including mechanical shaking for 3.5 hours), an equal volume of dried ether was added to the cooled mixture to precipitate a soluble part of the product. The precipitate combined with an insoluble part of the product was separated by centrifugation and freed from the adsorbed solvent *in vacuo*. The same procedure for the esterification was further repeated twice with the product, while the mixture was allowed to stand for 45 hours (including mechanical shaking for 4.5 and 6 hours) in the second treatment, and for 22 hourrs (shaking for 3.5 hours) in the third. The insoluble residue in the mixture diminished progressively as the treatment was repeated. Thus the crude esterified product was obtained in a yield of 393 mg.

To achieve complete esterification, a half of the crude product (198mg.) was shaken mechanically with aliquots (25,5,5, and 5 ml.) of unhydrous methanol and centrifuged, thus separated into a methanol soluble part and a small amount of a remaining insoluble one. This residue was again suspended in anhydrous methanol (10ml.) with the addition of concentrated HCl (0.1ml.) and left to stand for 28 hours at room temperature as described above. Then the mixture contained merely a negligible amount of an insoluble residue. The supernatant solution freed from the residue was combined with the clear solution of the methanol soluble part which had been separated in the above and further esterified for 28 hours with the addition of concentrated HCl (0.2 ml.). From the combined solution, the 4-times

esterified product was precipitated with ether and dried *in vacuo*. Yield, 187 mg.

Clupeine methylester hydrochloride obtained above dissolved into anhydrous methanol (20ml.), leaving no appreciable amount of an insoluble matter. To transform it into the sulfate, 2*N* H_2SO_4(1 ml.)was added under cooling to the methanol solution. The precipitate formed was then dissolved in water (10ml.)and reprecipitated with the addition of acetone* (70ml.) containing 2*N* H_2SO_4 (1 ml.). The ester sulfate obtained was freed from any trace of methanol, which might have been adsorbed, by the twice repeated precipitation from the aqueous solution with acetone containing H_2SO_4. Finally it was washed thoroughly with aliquots of acetone and dried *in vacuo*. The purified clupeine methylester sulfate was thus obtained as colorless fine powder in a yield of 176 mg.

(*b*) Under the same conditions of the esterification described in the above, samples of the less and the more soluble clupeine sulfate (500 and 300 mg., respectively) and the less soluble salmine sulfate (500mg.) were treated without repeat. In these cases, the mixtures were left to stand at room temperature for 72–92 hours and then the dried products (493, 296, and 497 mg., respectively), which had been combined with the precipitates formed by the addition of ether, were separated into the soluble and insoluble parts (the latters weighing 132, 146, and 236 mg., respectively) by the repeated extraction with methanol containing a slight amount of concentrated HCl and by the reprecipitation from the extracts with ether. Both parts of the products were purified by repeated precipitation from the aqueous solution with acetone containing H_2SO_4 as described above, and thus obtained as colorless glittering powder.

(*c*) Starting from the crude unfractionated clupeine and salmine picrates (200 and 400 mg., respectively), the suspensions in methanol (20 and 40 ml.) containing concentrated HCl (0.2 and 0.4ml.) were allowed to stand at room temperature for 43.5 and 47.5 hours, respectively. To the yellow mixtures containing insoluble residues an equal volume of ether were then added with cooling. The precipitates formed were, together with the residues, separated by centrifugation from the supernatant solutions containing picric acid liberated. The washed and dried products were again esterified for 45 and 46 hours, respectively. Now the insoluble residues**in the esterifying agents were removed (after dried *in vacuo*, 28 and 56 mg. of slightly brown powder, respectively), and then the colorless ester hydrochlorides were precipitated from the supernatants. These products were almost completely dissolved in methanol (10ml.), transformed into the ester sulfates and purified as described in the above. Thus the clupeine and salmine methylester sulfates (61 and 73 mg.) were obtained as colorless fine powder.

C. Esterification with Methanol Saturated with HCl—(*a*) According to the method by Felix and Mager (*4*) the crude unfractionated picrates of clupeine and salmine (152 and 400 mg., respectively) were suspended in anhydrous methanol (*ca.* 15 ml./g. picrate), and to these dried hydrogen chloride was introduced with cooling and shaking until saturation, while preventing from atomospheric moisture. After being left to stand at room temperature for 0.5–1 hour, the mixtures were treated with ether to precipitate the soluble fractions of the products. The crude esterified products thus obtained (95 and 123 mg.) were dissolved into small amounts of warm anhydrous methanol, and the insoluble residues**were removed (after dried, 10 and 48mg. as yellowish brown powder).

To the methanol solutions hydrogen chloride was again introduced for a few minutes

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* The clupeine sulfate precipitated from the aqueous solution with ethanol was found to contain *ca.* 2.2 per cent of C_2H_5OH (*14*).

** These appeared to be mainly composed of derivatives of the nucleic acid accompanied with the starting picrates.

until no more precipitation occurred. The precipitates formed (the main fractions) were separated from the supernatant mother liquors, from which another fraction of slightly yellow precipitate (10 and 25mg.) were obtained by the addition of ether. The main fractions were redissolved in warm methanol, and reprecipitated with 2.5 fold volumes of ether saturated with hydrogen chloride. The reprecipitation was repeated once more, and finally the colorless ester hydrochlorides of clupeine and salmine (64 and 46 mg.) were obtained. These were then transformed into their sulfates by repeated precipitation from the aqueous solutions with acetone containing H_2SO_4. The yields were 71 and 46 mg. for the clupeine and salmine methylester sulfates, respectively.

(*b*) The same procedure for the esterification was also applied to the less soluble clupeine sulfate (100 mg.). In this case, the methanol solution (4ml.) of the whole reaction product (96 mg.) in the first treatment, containing a small amount of an insoluble residue, was again introduced with hydrogen chloride as described above. The precipitate of the main fraction (81 mg.) was thus obtained, and the another fraction (12 mg.) was precipitated from the mother liquor with ether. The main fraction of the ester hydrochloride was almost completely dissolved in 3 ml. of warm methanol, purified and then transformed into the sulfate (87 mg.) as described in the above.

II. Determination of Methoxyl Content of Protamine Methylester Sulfates

Of the clupeine and salmine methylester sulfate preparation obtained in the above, the OCH_3 contents were determined by the micro volumetric method according to the principle of Vieböck and Brecher(*15*). The methyl iodide formed by treating the sample with hydriodic acid was transformed into iodic adid, which was titrated with KI and 0.01 *N* $Na_2S_2O_3$. The amount of consumed $Na_2S_2O_3$ was corrected for the blank determinations containing about the same amount of clupeine or salmine sulfate.* The contribution of the unesterified protamines to the blank values was sufficiently small to need the exact equivalent amounts of the protamines for the correction. The results are summarized in Table I.

III. Determination of Amino Nitrogen Content of Protamins Sulfates

The free amino nitrogen was determined on several specimens by the gasometric nitrous acid method with Van Slyke-Neill manometric apparatus(*16*). The specimens were two kinds of the purified preparations of the less soluble clupeine sulfate which were isolated from the different sources of fish obtained in 1947 and 1951, and two different preparations of the less soluble salmine sulfate from the same source of fish (1950) (*1*), as well as certain related amino acids. Under the given conditions by Van Slyke (*17*) various reaction periods were taken,including somewhat shorter or longer than that required for the complete reaction of α-amino acids at a given temperature (the volume of the reaction mixture, mainly 8ml.). The amount of the nitrogen gas evolved in the reaction was measured by the pressure it exerts at the volume of 2.0 ml. The total nitrogen contents of the samples were determined by the micro Kjeldahl method under the conditions of digestion recommended by Hiller *et al.* (*18*). A part of the results are shown in Table II.

RESULTS AND DISCUSSION

I. Methyl Esterification of Clupeine and Salmine and Their Molecular Weight

Methoxyl contents of seven kinds of clupeine methylester sulfates and

* The preparations were purified by repeated precipitation from the aqueous solutions with acetone. See the footnote cited before (* on p, 29).

four kinds of those of salmine, prepared by two different methods of esterification (method A and B) from three kinds of clupeine preparations and two

TABLE I

Methoxyl Content of Clupeine and Salmine Ester Sulfates and Their Molecular Weihgt Calculated as Free Bases

Starting substance[a] and method for esterification[b]	Fraction of reaction product	OCH_3 content of sulfate[c]	Molecular weight of free base[d]
Clupeine sulfate (less soluble) Method A, once	Soluble in methanol	0.23_6%	(10,500)
	Insoluble in methanol	0.14_4	(17,300)
Clupeine sulfate (more soluble) Method A, once	Soluble in methanol	0.23_1	(10,700)
Clupeine sulfate (less soluble) Method A, repeated	Soluble in methanol	0.39_8	6,200
Clupeine picrate (unfractionated) Method A, repeated	Soluble in methanol	0.41_0	6,100
Clupeine sulfate (less soluble) Method B	Soluble in methanol and insoluble in HCl-saturated methanol	0.41_6	6,000
Clupeine picrate (unfractionated) Method B	Soluble in methanol and insoluble in HCl-saturated methanol	0.46_3	5,400
Salmine sulfate (less soluble) Method A, once	Soluble in methanol	0.35_7	(7,000)
	Insoluble in methanol	0.14_6	(17,000)
Salmine picrate (unfractionated) Method A, repeated	Soluble in methanol	0.41_1	6,000
Salmine picrate (unfractionated) Method B	Soluble in methanol and insoluble in HCl-saturated methanol	0.39_1	6,400

(a) See the experimental part (*I*, *A*).

(b) Method A: Esterified once or repeatedly by allowing the starting substance to stand for a long time at room temperature in methanol containing 0.1*N* HCl. Method B: Esterified by a short time treatment in cold methanol saturated with HCl.

(c) Average value of 2–3 determinations, corrected for control.

(d) Calcurated as 80 per cent of the molecular weight of ester sulfate. Parenthesized values are not considered as molecular weight due to incomplete esterification.

kinds of those of salmine, respectively, and molecular weights of free protamines calculated therefrom are indicated in Table I. The calculation was made in consideration that 80 per cent of each ester sulfate consists of free protamine

base. The estimated value (20 per cent) for sulfuric acid content seems to be quite reasonable from the results reported by other investigators (*10*, *19–24*) and also preliminarily determined by ourselves.

In a case of esterification in which the starting material, purified clupeine sulfate (the less soluble fraction), was allowed to stand in methanol containing 0.1 *N* hydrochloric acid for 72 hours at room temperature according to the method by Fraenkel-Conrat (*8*), the methoxyl content of the purified ester sulfate obtained from a methanol (containing a slight amount of concentrated hydrochlorid adid) soluble fraction of the esterified product was 0.24 per cent, while that from an insoluble fraction was 0.14 per cent, as shown in Table I. In contrast with the above, in a case of repeated esterification under the same condition, until all the product dissolved completely into the esterifying agent (four times esterification, total reaction time for 138.5 hours), the separated product was clearly soluble in pure methanol (forming *ca.* 1 per cent solution) and had an increased methoxyl content of 0.40 per cent in the sulfate. Thus it is evident, that a treatment only once, under this condition is not enough for complete esterification of clupeine sulfate.* It is to be noted that a soluble esterified product obtained only by a single esterifiction under the same condition of the more soluble fraction of clupeine gave the same methoxyl content as that of the less soluble fraction (see Table I).

Separation of an esterified product from unesterified clupeine by making use of the solubility difference in methanol was tried in vain. Both the soluble and insoluble fractions in methanol always contained more or less a quantity of the methoxyl group. These results may be explained by supposing that the solubility difference in methanol between the sulfate and the hydrochloride is larger than that between clupeine and its ester. Thus it is probable that the methanol soluble fraction is contaminated with an unesterified substance which is dissolved as its hydrochloride, while the methanol insoluble fraction is contaminated with an ester as its sulfate. The same is true for salmine.

From these observations, the esterification reaction is expected to proceed more rapidly and completely, when a protamine salt soluble in the esterification reagent is used as a starting material. In fact, using crude picrates (unfractionated in the more and the less soluble parts) of clupeine and salmine as starting substances, the two times esterification products were found as expected to have the similar methoxyl contents as those obtained by the four times esterification of the purified, less soluble sulfate described before. This seems also to indicate that the less and the more soluble fractions of protamine have not been fractionated on the basis of their molecular weights.

In addition, we tried another esterification under more vigorous conditions,

* It had already been found in our preliminary experiment, that, without repetition of esterification, only an elongation of the period of esterification time did never cause an increase in the methoxyl group content to be introduced into clupeine (methoxyl contents of purified sulfate products obtained from the total reaction mixtures were 0.22, $0{,}27_5$, 0.23, and 0.23_5 per cent after being left to stand at room temperature for 1, 2, 7, and 26 days, respectively) (14).

to see whether the esterification of protamines above mentioned were complete or not. For this purpose the method by Felix (*4*) was adopted, in which the esterification was carried out by saturating gaseous hydrogen chloride under cooling into a suspension of protamine in methanol. Purified clupeine sulfate (the less soluble fraction) and crude picrates of clupeine and salmine, all of which are of the same preparations as used in the former esterification, were used as the starting substances. Esterification was repeated twice in each case. The ester sulfate obtained from a main fraction, which was soluble in a small amount of warm methanol and was precipitated by saturating hydrogen chloride under cooling, was found to have a methoxyl content practically not different, though somewhat fluctuating, from that of the product obtained before by repeated esterification with methanol containing 0.1 *N* hydrochloric acid at room temperature.

From the results above obtained, it can be concluded that almost complete esterification involving only the C-terminal carboxyl group of clupeine and salmine has been accomplished in methanol containing 0.1*N* hydrochloric acid. Thus the minimal molecular weight (6,000–6,200) calculated from the methoxyl content (0.40–0.41 per cent) of the ester sulfates, which were prepared under such conditions, may be considered to indicate true molecular weight. This can be supported by the value (5,400–6,400) obtained when a more drastic agent as methanol saturated with hydrogen chloride was used for esterification.

Furthermore, the molecular weight obtained above is similar to those obtained in our laboratory by other terminal group methods (5,600–7,100 for clupeine, 6,100–7,00 for salmine, each from the absorption measurements of their DNP-derivatives (*13*)*; 4,600 for the former and 4,400 for the latter from the results of their electrometric titration (*12*)). The value is also in accord with the results obtained in other laboratories of this institute by physicochemical measurements made on specimens from the same species of fish (5,000–6,000 by diffusion and viscosity (*25*), and *ca.* 5,000 by sedimentation and diffusion (*26*), for both clupeine and salmine; *ca.* 6,500 for clupeine by monomolecular film method**). These facts may also be considered to show, that the esterification has occurred almost completely without any detectable cleavage of the peptide chain.***

However, it is rather difficult to compare our results with those of the esterification of protamines obtained by foreign investigators,since consideration must be taken of some differences in species of fish material and in preparation

* Molecular weight of *ca.* 4,500–5,100 has been estimated in our laboratory concerning various specimens of the protamines by further absorption measurements at 360 mμ of the DNP-derivatives in *N* HCl (T. Ando and M. Yamasaki, unpublished data).

** K. Imahori, personal communication (1953).

*** The free amino nitrogen content of some of the specimens of the esters, which gave the values of molecular weight shown in the above, was similar to that of the unesterified protamine. Thus it has further been confirmed, that no cleavage of the peptide chain could have occurred in the treatment of esterification.

method. Extremely low methoxyl content (0.06 per cent) reported to be introduced in a commercial specimen of salmine sulfate by Fraenkel-Conrat and coworkers (*7*) can hardly be compared with our result, as no detailed description of the esterification conditions has been given. For the purpose of complete esterification of protamine sulfate according to their method, merely a one time treatment of the reaction seems to be insufficient, even when a condition of maximal esterification of various proteins as indicated by the American researchers (*8*) was adopted, and the reaction time was considerably prolonged (*14*). It seems rather necessary for that purpose to make repeated treatments, during which the sulfate may be successively transformed into the hydrochloride, dissolving then in the reaction agent.

The method of esterification by Felix *et al.* (*3*, *4*), using methanol saturated with hydrogen chloride, has been said to be so drastic as sometimes to lead to the cleavage of amide and peptide linkages, when applied to proteins (8, 27). Thus in each case of the experiment, some differences in the esterification condition and in the following fractionation method may often bring about variations of the methoxyl content in the products. This can be seen in our case where the variation of methoxyl values obtained by the method (B) is greater as compared with that obtained by another method (A) using methanol containing 0.1*N* hydrochloric acid. Methoxyl content of our clupeine methylester prepared by method B was found to be considerably lower than those* reported by Felix (*3*, *4*) and by Šorm (*5*), even when a difference of the content in sulfate and hydrochloride of the product was taken into account. One of the reasons for such a disagreement among three laboratories may consist in more or less difference of the esterification conditions adopted. It appears also probable as another reason, that specimens used for analysis by European researchers might content some methanol used for their purification, whereas our samples were carefully freed from methanol (see experimental part, II).

II. The N-Terminal Group of Clupeine and Salmine

Free amino nitrogen values obtained by manometric Van Slyke method on two kinds each of pure clupeine and salmine sulfate specimens are shown in Table II, together with those of arginine hydrochloride for comparison.

* Felix *et al.* (*3*) have first reported that the methoxyl content of each specimen of clupeine methylester hydrochlorides fractionated on the basis of its solubility in methanol saturated with hydrogen chloride was as follows; A_1: 3.20, A_2: 2.75, B (main fraction): 1.54 (molecular weight calculated 2,013), C: 0.76 per cent. Results reported later by the same authors (*4*), employing a little modified condition of esterification, were A: 1.85, B: 0.96, and C (main fraction): 0.65 per cent (molecular weight caluculated 4,770). However, values obtained by Šorm (*5*) using the unmodified method by Felix (*3*) were A_1: 1.10, A_2: 1.66, B: 1.55, and C: 1.13 per cent; also C: 0.83 per cent (molecular weight calculated 3,740) (*28*) by the modified method (*4*, *27*); molecular weights calculated from diffusion-viscosity measurements (*6*) were 4,400 (or 5,200) for clupeine A and 4,200 (or 4,900) for C, and also the value of the same order for B.

Van Slyke (*17*) has given a time necessary for amino acids to react completely with nitrous acid under a definite condition, *e.g., ca.* 4 minutes at 20°.

TABLE II

Amino Nitrogen Content of Clupeine and Salmine Sulfates

Substance[a]	Sample Weight	Sample Total-N	Reaction Temp.	Reaction Time[b]	Found Amino-N	Amino-N[c] / Weight	Amino-N[c] / Total-N
	mg.	*mg.*	°	*min.*	*mg.*	*Per cent*	*Per cent*
Clupeine sulfate (1947)	98.0_5	23.5	20	3	0.179	0.18	0.76
	98.0$_5$	23.5	20	*5*	0.162	*0.16$_5$*	*0.69*
	39.2	9.41	20	15	0.061_5	0.16	*0.65*
	11.4	2.72	20	*4.5*	0.027_5	*0.24*	*1.01*
	11.4	2.72	20	*5*	0.025	*0.22*	*0.91*
Clupeine sulfate (1951)	35.1	8.06	16	*6.5*	0.048	*0.14*	*0.59*
	35.7	8.16	16	*6.5*	0.050_5	*0.14*	*0.62*
	17.5	4.03	16	15	0.028	0.16	0.70_5
	17.8_5	4.08	16	15	0.029	0.16	0.70
Salmine sulfate (1950)	61.9	14.4	20	3	0.018	0.029	0.12
	61.9	14.4	20	*5*	0.027	*0.004*	*0.19*
	49.5	11.5	20	15	0.018_5	0.037	0.16
Salmine sulfate (1950)	38.8	9.26	16	*6.5*	0.016	*0.041*	*0.17*
	19.4	4.68	16	15	0.009_5	0.049	0.20_5
Arginine-HCl	4.75	1.22	21.5	*4*	0.316	*6.65*	*25.9*
	4.75	1.22	16	*6*	0.322[d]	*6.78*[d]	*26.4*[d]
	4.75	1.22	16	6.5	0.323	6.79	26.5
	4.75	1.22	16	15	0.322	6.77	26.4

(a) The specimens of clupeine sulfate, the less soluble fraction, were prepared from the different sources of fish obtained in 1947 and 1951. Those of salmine sulfate are of the different preparation from the same source of fish (1950) (*1*).

(b) Such a reaction time is shown italicized as is longer than but nearest to the srandard time given for amino acids by Van Slyke. Both in such a period of time and in 15 minutes, 100.8 ± 0.8 per cent of the total nitrogen reacted as amino nitrogen for alanine and 0.75 ± 0.01 per cent for proline.

(c) Available values are shown italicized.

(d) Average value of 3 determinations; found amino nitrogen is within a range of ± 0.001mg.

As seen from the table, amino nitrogen value of protamines at 15 minutes reaction are in most cases somewhat higher* but in some cases lower than

* A similar tendency to have higher values at 15 minutes reaction than those of the standard conditions has also been found by our measurements on amino nitrogen of peptides containing arginine, though the result are not listed in Table II.

those obtained at the time given for amino acids by Van Slyke. These deviations from the values of the standard conditions may be considered to be within a range of experimental error. It appears therefore that all the free amino groups of protamine reacted quantitatively under the standard conditions given for amino acids by Van Slyke.

As described before, no lysine was found in our specimens of clupeine and salmine, judging from the result of their amino acid composition analysis (*1*). Accordingly, the free amino nitrogen found in the above can first be expected to occur from N-terminal groups of protamine molecules. However, it must be considered that nitrogen of so many guanidyl groups in the molecule (*ca.* 28 groups per molecular weight 6,000) may contribute more or less to its amino nitrogen value, since *ca.* 88 per cent of the total protamine nitrogen is occupied by arginine nitrogen (*1*, *12*). Estimation of the extent of contribution from guanidyl nitrogen to amino nitrogen of the standard conditions was attempted by an extrapolation method but was found to be difficult on account of its experimental error. From the measurement on α-benzoyl-arginine amide by Doherty and Ogg (*29*) using a modified apparatus for insoluble proteins, it can be calculated that guanidyl groups in our specimens of protamines give 0.20 per cent of their total nitrogen as amino nitrogen after 5 minutes reaction at 22–23°. This value of amino nitrogen lies in just the same order as that (0.18 per cent) found in our salmine under the standard conditions. We have already found by the DNP-method, that almost all of the N-terminal residue of our salmine is occupied by proline, an α-imino acid (*11*, *30*). Hence, most of the part of the amino nitrogen value found in salmine is considered to be due to its guanidyl groups. Even in a case in which salmine might be inhomogeneous with respect to its N-terminus and have any other terminal amino group, the content of molecules having N-terminal other than proline would be supposed to be extremely low.*

Since clupeine has been found to have similar nitrogen and arginine contents (*1*, *12*) and molecular weight as those of salmine, the amino nitrogen value (0.18 per cent) found of salmine may be corrected for clupeine as a contribution from the guanidyl groups to the amino nitrogen value. Then there remains in clupeine yet 0.7 per cent of the total nitrogen (0.87—0.18= 0.69, or corresponding to $0.69\times24/100=0.16_5$ per cent per weight of specimen) or 0.4 per cent (0.61—0.18=0.43, or 0.10 per weight) as a free amino nitrogen, found under the standard conditions for two kinds of the clupeine sulfate specimens. This could result from its N-terminal amino group. Thus tentative calculation based on the amino nitrogen value gives a molecular

* Supposing the molecular weight of the protamine to be 6,000, the contribution of imino group to amino nitrogen is only 0.006 per cent of the total nitrogen and may be negligible (see the footnote of Table II). When all of the amino nitrogen found is assumed to be due to the contaminated N-terminal amino group, the content in salmine of contaminated molecules with the N-terminal amino group would be calculated to be 23 per cent in a similar way as in clupeine which is described below. The value of the content above obtained, is quite inconsistent with our result by the DNP-method.

weight of *ca.* 8,500 or 14,000 for clupeine sulfate. Considering the sulfuric acid content to be 20 per cent as described in the preceding section, a molecular weight of 7,000 or 11,000 is given for free clupeine.* This value of molecular weight is considerably higher when compared with that (6,000) obtained from the free carboxyl group determination as already described. From the disagreement between them, contamination of the molecules with imino group in N-terminus can be anticipated. Indeed, as explained recently by various careful analyses of DNP-derivatives of our clupeine (*30*), it has been shown, that specimens of our clupeine are inhomogeneous concerning their N-terminal residues, having proline besides alanine in various extents (the proline content is *ca.* 10–25 per cent in 1947 preparation and *ca.* 50–60 per cent in 1951). Assuming that an amino group of alanine in the N-terminal reacts quantitatively in Van Slyke determination, while an imino group of proline does not at all**, and that both kinds of molecules with alanine or proline in their N-terminus have the same molecular weight (6,000), the N-terminal alanine content would be calculated from the amino nitrogen values to be 90 per cent for a specimen of clupeine obtained in 1947 and 50 per cent for that in 1951. These results are not contradictory to those obtained by the DNP-method referred to in the above.

Amino nitrogen values*** found in specimens of clupeine and salmine by Waldschmidt-Leitz (*19*, *31*, *32*) and by Fraenkel-Conrat (*7*) are in the same order as in ours and may be explained in a similar way, though there must be considered some differences among specimens used by these three laboratories.

As a conclusion derived from the results of determination of free carboxyl group by its methyl esterification and of the free amino group by manometric Van Slyke method, each of our specimens of clupeine and salmine seems to consist of an open chain polypeptide of the same order of molecular weight (6,000). Clupeine seems to be inhomogeneous regarding its N-terminal group, having an amino group in half or more parts of molecules and the imino one in the remaining part. Almost all the N-terminal groups of salmine, however, appear to be composed of the imino group.

SUMMARY

1. Methylesters of clupeine and salmine were prepared from purified

* Molecular weight of 6,400 for clupeine formerly reported in brief (*12*) was a value derived from a tentative calculation from amino nitrogen values (0.18 and 0.16_5 per cent amino-N/weight) in 3 and 5 minutes reaction (see Table II) of a specimen obtained in 1947, without any consideration of an effect of guanidyl groups. For salmine, those values (0.029 and 0.044 per cent) were adopted in that time.

** See the preceding footnote in p. 36 and also that in Table II.

*** Free amimo nitrogen/total nitrogen has been reported to be 0.96, 0.80 or 0.82 per cent for clupeine and 0.42 per cent for salmine by Waldschmidt-Leitz (*19*, *31*, *32*), and that per weight of salmine to be *ca.* 0.1 per cent by Fraenkel-Conrat (*7*).

sulfates and crude picrates of the protamines (derived from *Clupea pallasii* and *Oncorhynchus keta*, respectively) by two methods, in which esterification was carried out (A) by allowing them to stand at room temperature for prolonged periods of time in methanol containing 0.1*N* hydrochloric acid, or (B) by short time treatment under cooling in methanol saturated with hydrogen chloride.

2. Methoxyl contents of the purified ester sulfates of both protomines were found to be 0.40–0.41per cent in the products obtained by method A when esterified thoroughly, and 0.39–0.46 per cent by method B. Since these methoxyl groups are considered to be introduced only into C-terminal carboxyl groups of the protamine molecules, molecular weight of 6,000–6,200 (or 5,400-6,400 by method B) was calculated for both protamines from the result.

3. Free amino groups (N-terminal groups) of clupeine and salmine were determined by manometric Van Slyke method. Consideration was given to an amount of contribution from the guanidyl groups to the results. Thus clupeine was found to have an 0.5–0.9 equivalent of the amino group per molecule (molecular weight=6,000), the remaining part appearing to be an imino group. Salmine was found to contain almost none of the amino group, hence it was supposed to have mainly an imino group in the N-terminus.

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EFFECTS OF SOME INHIBITORS ON THE METABOLISM OF GUINEA PIG BRAIN SLICES

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It is a generally accepted hypothesis that the functional phenomena observed in nervous tissue are causally related to its metabolic activities. Of course, some aspects of functional phenomena in nervous tissue are possibly regarded as purely biophysical events. At any rate, we must ask ourselves the question how far are the nervous phenomena dependent on cell equilibria as regulated by enzymatic activities. One of the most fruitful methods on this line is by the use of metabolic inhibitors whose range of action is rather well known. Practically there have been many investigations intending to clarify the causal relations between the function and the metabolism in nervous tissue using some inhibitors (*1*, *2*).

But, generally speaking, the purer the enzyme preparation, the greater is the specificity of inhibitory action of a poison, and when a metabolic poison is applied to the intact tissue its action is likely to be much smaller and less specific. Therefore, we should consider on the factors operating in the reactions between poisons and enzymes especially in the intact tissue, and on the limitations of these approach in case of understanding the practical experimental results. Generally, the metabolic activity of nervous tissue are very difficult to measured in the intact conditions. Most easily available preparation for the measurement of metabolic activity of nervous tissue is cerebral cortex slices of mammals. It is thought to be possible by the use of brain slices, prepared without disrupting the majority of cell structures, to observe the mode of actions of metabolic poisons on the organized nervous tissue, to clarify its metabolic characteristics, and further to get some bases for the considerations on the dependency of functional events upon the metabolic activities in nervous tissue.

METHODS

Preparation and Incubation of Brain Slices—Guinea pigs weighing from 200 to 250 g. were used exclusively. Preparation and incubation of cerebral cortex slices were the same as described in previous reports (*3–5*). Brain slices were always incubated under pure oxygen at 37° in Krebs-Ringer phosphate solution of pH 7.0. All the metabolic inhibitors used were of commercial "guaranteed reagent grade" and were neutralized to pH 7.0 with NaOH just prior to use. They were added in advance to the incubating medium.

Analytical Methods—Oxygen uptake was measured by means of conventional manometric

techniques (*6*). At the end of the incubation period (90 minutes), aliquots of the medium were rapidly transferred to trichloroacetic acid, and the deproteinized supernatant was used for the chemical analyses. Glucose utilization was estimated using the "anthrone" reagent (*7*), lactic acid formation by the method of Barker and Summerson (*8*) or of Hullin and Noble (*9*), and pyruvic acid by the method of Friedemann and Haugen (*10*). When glucose was used as a substrate and the pyruvic acid accumulation was to be estimated, the total hydrazone method was employed. When sodium pyruvate was used as a substrate, the pyruvic acid utilization was determined by the general extraction method with xylene or by the simpler total hydrazone method. Both of the determined values by these methods were thought to be strictly due to pyruvic acid, judged according to the solubility nature of its dinitrophenylhydrazone (*11*).

TABLE I

Effects of Inhibitors on Metabolism of Glucose in Guinea Pig Brain Slices

Inhibitor and its concentartion	Oxygen uptake *μ moles/g./hour*	Lactate formation *μ moles/g./hour*	Glucose utilization *μ moles/g./hour*	Pyruvate accumulation *μ moles/g./hour*	Glucose oxidized (calculated) *μ moles/g./hour*
None	56.6±5.5 (13)	17.1±2.6 (13)	18.1±2.1 (13)	——	9.6
Malonate 5mM	46.5±3.0 (6)	16.6±2.0 (6)	15.8±1.7 (6)	——	7.5
Malonate 10 mM	42.9±2.1 (8)	24.7±4.0 (7)	20.5±2.7 (7)	——	8.2
Azide 0.3 mM	57.3±4.4 (6)	28.5±0.9 (6)	22.1±1.0 (6)	——	7.9
IAA 0.03 mM	54.6±3.0 (6)	15.5±2.6 (6)	14.6±1.8 (6)	——	6.9
Fluoride 2 mM	51.2±4.0 (6)	8.8±0.8 (6)	11.1±1.7 (6)	——	6.7
Fluoride 5 mM	46.1 (2)	7.4 (2)	10.5 (2)	——	6.8
Fluoride 10 mM	34.4 (2)	8.5 (2)	7.1 (2)	——	2.9
Arsenite 0.03 mM	50.2±3.7 (6)	25.9±2.5 (6)	25.0±2.2 (6)	10.1±1.3 (6)	7.1
2-4 DNP 0.05mM	136.2±12.0 (4)	36.8±2.8 (4)	40.9±2.5 (4)	6.4±1.0 (4)	19.3
KCl 100 mM	94.4±8.1 (4)	59.4±5.9 (4)	51.0±4.2 (4)	——	21.3
Malonate 10 mM +KCl 100 mM	36.9±1.8 (6)	68.4±1.6 (6)	51.2±3.3 (6)	——	17.0

Substrate: glucose, 3.3 mM. Medium: Krebs-Ringer phosphate solution, pH 7.0.

Inhibitor added in advance to the medium.

All the results obtained were expressed as μmole per gram of wet weight of slices per hour. Mean and standard deviation were given. Numbers of the experiments were shown in parentheses in the Tables.

RESULTS

1. Effects of Inhibitors on Glucose Metabolism

Effects of the well known inhibitors of glucose metabolism on the oxygen uptake, lactic acid formation and glucose utilization in guinea pig cerebral cortex slices with glucose as a substrate were tested. As seen from Table I,

at a smaller concentration, malonate, azide, iodoacetate, fluoride and arsenite did not depress the oxygen uptake significantly or at all, but the amount of glucose consumed oxidatively, calculated from the glucose utilization and lactic acid formation, was rather decreased clearly. When the concentration of inhibitors applied was increased, they lowered apparently the oxygen uptake and also did the calculated glucose oxidation.

Generally speaking, the pattern of the effect of malonate was similar to that of either azide or arsenite. They increased the lactic acid formation and glucose utilization at such a concentration that their inhibitive effects on the oxygen uptake were not significantly observed. These observations were only the confirmation in case of azide (*12*). On the contrary, iodoacetate and fluoride decreased both the lactic acid formation and glucose utilization at samller concentration.

Next, the effect of malonate on the cerebral cortex slices in relation to the potassium effect was studied. As already observed by Kimura and Niwa (*13*) and Heald (*14*), the respiration of brain slices in the normal

TABLE II

Effect of Malonate on Metabolism of Glucose in Guinea Pig Brain Slices

Medium	Malonate	Oxygen uptake *μ moles/g./hour*	Lactate formation *μ moles/g·/hour*
Glycylglycine buffer	0	56.3±4.8(5)	17.4±3.8(5)
	10mM	34.5±2.4(5)	38.0±1.1(5)
Phosphate buffer	0	56.2±4.3(5)	15.9±2.2(5)
	10mM	40.8±3.5(5)	24.7±2.2(5)

Substrate: glucose, 11 mM. Medium: Krebs-Ringer glycylglycine buffer solution or Krebs-Ringer phosphate buffer solution of pH 7.0.

control condition was rather insensitive to malonate at a concentration of 10 mM, but in the potassium effect, malonate inhibited completely the augmented oxygen uptake. Contrary to these observations, 10 mM malonate inhibited the oxygen uptake and increased the lactic acid formation in absence of the potassium effect statistically significantly in the present experiments. These effects of malonate were not observed to be changed even when glycylglycine was used instead of phosphate as a buffer in the medium. When the concentration of glucose as a substrate was raised to 11 mM or glycylglycine was used as a buffer, the increase of lactic acid formation thereby was rather predominant. In presence of the potassium effect, malonate inhibited completely the augmented oxygen uptake and rather increased the lactic acid formation, but the glucose utilization was not changed at all.

In the presence of malonate, the oxygen uptake of cerebral cortex slices was similarly maintained steadily as in the absence of poison for more than one hour at a lowered and inhibited rate (Fig. 1). However, the endogenous respiratory rate of them was not maintained steadily, but descreased gradually

with time. Although the endogenous respiration of brain slices is rather insensitive to a concentration of 10 mM of malonate (*3*), the content of oxygen uptake in the presence of malonate may not be same as that of the endogenous respiration itself, but may be of the composite nature.

Concerning to the effect of malonate, its effect in the further addition of fumarate besides glucose as substrate to the medium was tested. The addition of fumarate to the medium seemed to abolish the inhibitive effect of malonate

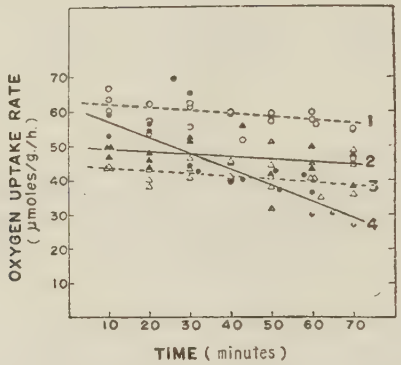

Fig. 1. Oxygen uptake rate of guinea pig brain slices.

Guinea pig cerebral cortex slices incubated under pure oxygen at 37°. Incubation medium; Krebs-Ringer phosphate solution of pH 7.0. Substrate or inhibitor added as follows; 1: glucose, 3.3mM; 2: glucose, 3.3mM plus malonate, 5 mM; 3: glucose, 3.3 mM plus malonate, 10 mM; 4: no addition (endogenous respiration).

Table III

Effect of Adding Fumarate on Respiration of Guinea Pig Brain Slices

Condition			Oxygen uptake *μ moles/g·/hour*
Glucose+fumarate			86.4±8.2 (3)
//	//	+KCl	145.2±15.6(3)
//	//	+malonate	78.1±12.4(4)
//	//	+KCl+malonate	84.5±6.0 (4)

Substrate: glucose, 11 mM; fumarate, 20 mM; malonate, 10 mM. Medium: Krebs-Ringer phosphate solution of pH 7.0. KCl or malonate added in advance to the medium.

on the oxygen uptake of brain slices (Table III). But in the presence of excess potassium, the effect of malonate were similarly observed as in the absence of fumarate.

The effect of 2, 4-dinitrophenol (DNP) on the cerebral cortex slices has already been reported by several authors (*15*, *16*). As well known, 0.05 mM of DNP increased both the oxygen uptake and the lactic acid formation of

brain slices and also increased the glucose utilization. Under any conditions used except in the iufluence of DNP and arsenite, pyruvic acid accumulation in the medium was never detected by the present method, probably it might be below 1.0 μ mole/g·/hour. By the addition of arsenite or dinitrophenol, pyruvic acid was exceptionally accumulated and measurable.

II Effects of Inhibitors on Pyruvate Metabolism

In the absence of inhibitors, pyruvate maintained the oxygen uptake of

TABLE IV

Pyruvate Utilization and Lactate Accumulation in Guinea Pig Brain Slices

Condition	Oxygen uptake μ moles/g·/hour	Pyruvate utilization μ moles/g·/hour	Lactate accumulation μ moles/g·/hour
Control	63.4±7.2(5)	43.9±4.6(4)	10.1±0.8(5)
Fumarate, 2.5 mM added	79.0±3.0(8)	56.5±3.3(6)	11.5±0.6(8)
KCl, 10 mM added	116.6±11.0(6)	65.4±3.4(4)	5.9±0.7(6)
DNP, 0.05 mM added	102.7±10.7(6)	55.9±5.8(6)	6.9±0.5(6)

Substrate : sodium pyruvate, 6.0mM. Medium : Krebs-Ringer phosphate solution of pH 7.0. Pyruvic acid measured by the general extration method and lactic acid by the method of Hullin and Noble (*9*).

TABLE V

Effects of Inhibitors on Metabolism of Pyruvate in Guinea Pig Brain Slices

Inhibitor and its concentration	Oxygen uptake μ moles/g·/hour	Pyruvate utilization μ moles/g·/hour
None	56.4±7.1(5)	41.2±4.3(5)
Malonate, 10 mM	49.0±3.7(6)	38.2±1.3(6)
KCl, 100 mM	98.8±9.2(3)	52.7±2.9(3)
KCl, 100 mM+malonate, 10 mM	73.3±6.6(6)	44.1±2.8(6)
Azide, 0.3 mM	58.3±7.4(4)	42.3±1.3(3)
DNP, 0.05 mM	76.5±8.2(4)	42.1±3.7(4)

Substrate: sodium pyruvate, 6.0 mM. Medium: Krebs-Ringer phosphate solution of pH 7.0. Pyruvic acid measured by the total hydrazone method.

cerebral cortex silces at a fairly high level as glucose did. Added pyruvate was consumed at a rate of about 40 μ moles/g·/hour (Tables IV and V), but the lactic acid accumulation was rather small. The usual method of determining lactic acid was not satisfactory in the presence of excess pyruvic acid. Therefore, in the present experiments (Table IV), the method of Hullin and Noble (*9*) was used. By the addition of small quantity of

fumarate (2.5 mM), both the oxygen uptake and the aerobic pyruvate utilization of cerebral cortex slices were increased clearly. In the presence of excess KCl, or of DNP, the oxygen uptake and pyruvate utilization was increased, but the lactic acid was less accumulated.

A concentration of 10 mM of malonate seemed to be inhibitory to the oxygen uptake and pyruvic acid utilization of brain slices when pyruvate was used as a substrate (Table V). Under the effect of potassium, the effect of malonate on the pyruvate metabolism was similarly observed. But the residual oxygen uptake in the presence of both excess potassium and malonate was considerably higher than that in the control medium.

Azide, at a concentration that promoted the lactic acid formation from glucose, had no effect on the pyruvate metabolism. Between the results presented in Tables IV and V, there was found to be a difference concerning to the effect of DNP on the pyruvic acid utilization. Data in Table IV were obtained using the general extraction method for the determination of pyruvic acid, but ones in Table V by the total hydrazone method. Except the above, all of the experimental arrangements were intended to be quite same. Therefore, the cause of this difference was almost mysterious to the authors. The experiments presented in Teble V were undertaken from May to June, but the ones in Table IV from November to December. These seasonable difference might be among the causes of difference in the results.

DISCUSSION

Generally it may be believed that glucose is the usual fuel for the nervous tissues, in brain (*17*) and also in ganglion (*1*). In nervous tissue, as in many other tissues, glucose may be thought to be metabolized aerobically through the glycolytic or Embden-Meyerhof pathway and the citric acid cycle. Weil-Malherbe (*20*) and Krebs (*21*) pointed out many evidences for the possible operation of glycolytic route and of citric acid cycle in the brain. Many investigations have already been reported on the effects of inhibitors on the metabolism of glucose in cerebral cortex slices intending to certify the metabolic pattern of nervous tissue (*12–14*). But the considerations resting upon the effects of metabolic inhibitors on brain slices seemed to be complicated and problematical. Metabolic inhibitors, widely used as poisons for glucose metabolism and also tested in the present experiments, are known to act more or less specifically upon an enzyme. But when applied to the organized multienzyme system, such as slice preparation, their mode of action may be quite different as seen from the results mentioned above and those obtained by other authors. Therefore, in many cases, the conclusions resting only upon the experiments using the metabolic inhibitors in the intact tissues might be thought to be questionable and have gone too far.

It is said that the oxygen uptake of brain slices under normal conditions is rather resistant to azide (0.1 mM) and malonate (10 mM), but that under the effect of potassium more susceptible to the same inhibitors (*12–13*). And further, because it seems to be justified to consider that the potassium effect

represents one of the metabolic characteristics of brain tissue in excited state, azide or malonate could be said to distinguish the resting metabolism as opposed to the active one in brain tissue.

However, as clearly shown in the preceding pages, at a concentration of 10 mM, the inhibitive effect of malonate on the glucose metabolism in brain slices was clearly significant statistically, and judging from the glucose oxidation calculated basing upon the glucose utilization and lactic acid formation, malonate was similarly inhibitive in absence of the potassium effect as well as in presence of it. Furthermore, in view of the fact that the endogenous respiration is fairly resistant to malonate, the conclusions resting only upon the ineffectiveness of malonate on the observed oxygen uptake that the citric acid cycle may not be the prevailing pathway in the resting brain might be thought to be quite questionable.

At any rate, all the experimental facts mentioned above and those observed so far (*5*, *22*, *23*) seem to be rather in favor of the general operation of citric acid cycle in the brain slices under the normal conditions. Probably the similar discussion may be applied also upon the effects of azide on the brain slices.

The understanding on the effects of 2, 4-DNP on brain tissue has been quite controversial and there have previously been many investigations upon them (*15*, *24*, *25*). As seen from the results mentioned above, DNP, at a concentration of 0.05 mM, increased the oxygen uptake, lactic acid formation and glucose utilization. At the same time it accumulated pyruvic acid in the medium as well as arsenite did. Although the latter effect was rather naturally understandable from the enzyme known to be attacked thereby, the former was difficult to be explained from the facts observed so far. Of course, these effects could not be explained from the fact that 2, 4-DNP uncouples the oxidative phosphorylation (*26*).

In the effects of inhibitors tested on the metabolism of pyruvate in brain slices, any clear evidence against the authors' consideration may not have been pointed out, but rather they may be confirmative. Resting upon these facts and considerations, it is the authors' belief that the experiments using only the metabolic inhibitors cannot give any decidal conclusion on the metabolic route, especially when they are applied to the intact tissues. It is wise not to go too far basing only upon these experimental results and rather to wait for further investigations through the other channels.

SUMMARY

The effects of inhibitors on the oxygen uptake, lactic acid formation and glucose utilization by guinea pig brain slices metabolizing aerobically with glucose as a substrate were studied. Inhibitions of oxygen uptake of brain slices under the normal conditions by some inhibitors were not so predominant, although under the effect of potassium they were very clearly observed. But if considered resting upon their effects on lactic acid formation and glucose utilization, the differences of the metabolic characters between in presence

and in absence of the potassium effect were not clearly confirmed.

Many complicated factors to be considered upon understanding the results obtained in the brain slices using metabolic inhibitors were mentioned and discussed. From the considerations cited above, it might be thought that glucose would be metabolized aerobically through the glycolytic and citric acid cycle routes either in presence or in absence of the potassium effect in brain slices as seen in many other tissues.

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STUDIES ON AN AMYLASE OF CANDIDA TROPICALIS VAR. JAPONICA

I. MALTASE AND TRANSGLUCOSYLASE ACTIVITIES OF THE AMYLASE

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In a previous communication (*1*), it was reported that *Candida tropicalis var. japonica*, a wild yeast, had two maltose-splitting enzymes, one of which was designated as acid α-glucosidase because it had an optimum pH of about 4.0 and was also able to hydrolyze methyl α-glucoside. On the other hand, Yamaguchi, who isolated this yeast, had briefly reported the presence in it of an amylase (*2*). A later experiment of the present author gave rise to the suspicion that this amylase was identical with the acid α-glucosidase. The experiment further showed that the acid α-glucosidase preparation from this yeast formed other oligosaccharides when acting upon maltose. These findings led to the hypothesis that the amylase of *Candida tropicalis var. japonica* was one of unexampledly broad specificity hydrolying not only starch and maltose but also hetero α-glucosides, and acting, moreover, as a transglucosylase. The present paper deals with the experiments that were done to verify this hypothesis, However, perfect purification or crystallization of the supposed enzyme has so far not been achieved since, as in the case of yeast invertase, the mannan contained in the enzyme preparation persistently accompanied the source of the above enzymic activities throughout all attempts to purify it. Therefore, the means adopted to make clear whether or not amylase, maltase and transglucosylase activities were those of a single enzyme protein was to examine the ratio of these activities after various treatments of the enzyme preparation.

METHODS

Preparation of Enzyme Solution—The yeast was grown by the method described in the previous paper (*1*), but as carbon source, unless otherwise stated, commercial dextrin, which gave maximal enzyme production, was used. The autolyzate was also obtained as previously described (*1*), except that the preparation was allowed to stand at 37° throughout. It was cleared by acidifying to pH 3.5 with 4 *N* HCl and filtering. The precipitate obtained by adding to the autolyzate the same volume of ethanol was directly, or after drying *in vacuo* for storage, dissolved in distilled water and dialyzed against tap and distilled water successively. This was called "crude enzyme solution," and was devoid of sucrase, cellobiase and so-called α-amylase activities. It contained so much yeast mannan that the nitrogen content was only about 1.3 per cent of its dry weight. The mannan was largely removed from the crude enzyme solution when the latter was loaded on a

Duolite C 10 column and eluted (Fig. 3). This eluate, which was called "resin-eluted enzyme solution," has been used in most of the experiments described in this paper.

Assay Procedures—All enzyme reactions were carried out by incubating tubes at 37° for 20 hours, containing reaction mixtures and a few drops of toluene and covered at tops with vinyl cloth. The reaction mixture for the assay of amylase or maltase activity consisted of 2 ml. of 2 per cent soluble starch solution or *M*/25 maltose solution respectively, 1 ml. of McIlvaine buffer (*M*/10 citric acid and *M*/5 disodium phosphate), of pH 4.0 unless otherwise specified, and 1 ml. of enzyme solution. To stop the reaction, 12 ml. of 1 per cent sodium carbonate solution were added to each tube. Using a part of the mixture the increase in reducing power was determined by Somogyi's method (*3*) and per cent hydrolysis of the substrate calculated. Transglucosylase activity was observed according to the method worked out by Takano and Miwa (*4*) which is based the transfer of glucosyl radical from phenyl α-glucoside to glycerol. The reaction mixture consisted of 0.5 ml. of *M*/12.5 phenyl α-glucoside, 0.5 ml. of McIlvaine buffer of pH 4.0 unless otherwise specified, which was 1 *M* in glycerol, and 1 ml. of enzyme solution. To stop the reaction, 5 ml. of 1 per cent sodium carbonate solution were added. Using 5 ml. of the resulting mixture, the increase in reducing power was determined by Somogyi's method and expressed as per cent of reducing sugar liberated on that potentially available from the donor. The released phenol was determined, after adequate dilution of 1 ml. of the residual mixture, by light absorption at 287.5 mμ in *N*/10 NaOH and per cent of phenol liberated on that potentially available from the donor was calculated. The difference in per cent between these two values was regarded as transfer per cent.

Yeast mannan contained in the enzyme solution was determined by Somogyi's method after refluxing it in *N* HCl for 2.5 hours and expressed as mg. of glucose per ml. of fraction.

Standard Curves—Standard curves for amylase, maltase and transglucosylase activities were constructed by assaying variously diluted crude enzyme solutions and by plotting activity against relative concentration of enzyme solution, as shown in Fig. 1. The activity of a given enzyme solution can be obtained from the curves in terms of units per ml. by assaying it according to the above procedures after aequate dilution where necessary. If the three enzyme activities are those of a single enzyme protein, their relative activity ought to be essentially constant, whatever the treatment to which the enzyme solution may be subjected.

RESULTS

pH-Activity Curves—Using McIlvaine and citrate-HCl buffer, the effect of pH on the amylase, maltase and transglucosylase activities of the resin-eluted enzyme solution was investigated. The pH-activity curves obtained are shown in Fig. 2. The optimum pH for all three activities is 4.0, though the slopes of the curves are somewhat different from each other. Phosphate can be demonstrated not to take part in transglucosylation from the fact that the transfer per cent in the reaction mixtures lacking phosphate is not less than in the mixtures containing McIlvaine buffer, as seen in Fig. 2.

Gradual Elution from Resin Column—Duolite C 10, a cation exchange resin (100–200 mesh), equilibrated beforehand with McIlvaine buffer, pH 3.4, was introduced into a 25 ml. biuret tube (inside diameter, 1.0 cm.) to a height of 12 cm. 4 ml. of the crude enzyme solution, prepared by dissolving dried ethanol-precipitate powder (2 per cent) in McIlvaine buffer of pH 3.4, was applied to the column and eluted with the same buffer of pH 3.4 and 5.4 successively at a rate of 2 drops per minute. The effluent was collected

in 6 ml. fractions. The three enzyme activities and the amount of yeast mannan per ml. of each fraction were measured. The results are illustrated in

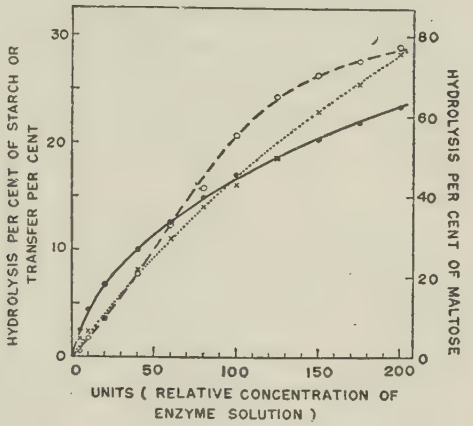

FIG. 1. Standard curves for obtaining units per ml. of amylase, maltase and transglucosylase activities. For the assay procedures and directions for use, see the text. ●—— Amylase activity; ○—— maltase activity; ×····· transglucosylase activity.

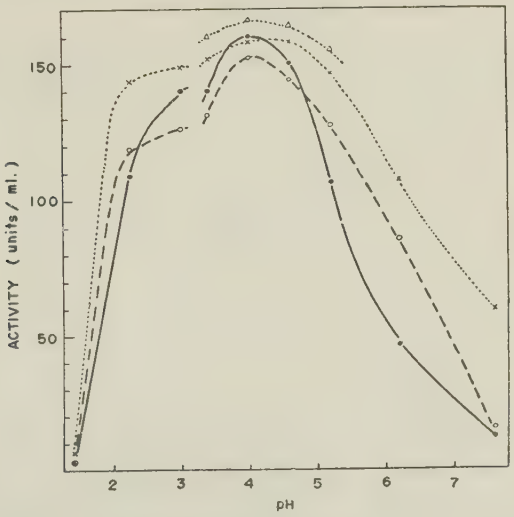

FIG. 2. Effect of pH on amylase, maltase and transglucosylase activities of the resin-eluted enzyme solution. Citrate-HCl and McIlvaine buffers were used in the lower and upper pH range respectively. ●—— amylase activity; ○—— maltase activity; ×····· transglucosylase activity. (△····· Transglucosylase activity when acetate buffer was used.)

Fig. 3, which shows that the three enzyme activities emerged in two peaks, as was observed in the experiment with yeast invertase (*5*), but were distributed

almost in parallel throughout. It is also seen in Fig. 3 that the major part of the yeast mannan passed straight down the column with the first eluting buffer and that the major part of the activities appeared in the second peak, considerably purified in regard to yeast mannan.

Heat Treatment—Four tubes, each of which contained 4 ml. of the resin-eluted enzyme solution, were heated for 10 minutes in water baths of 50°,

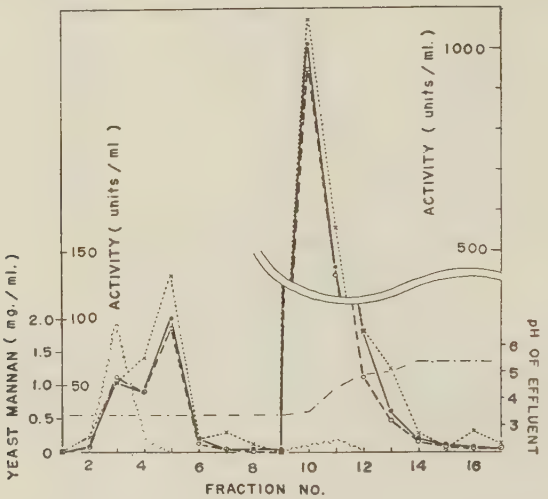

Fig. 3. Distribution of three enzyme activities and yeast mannan in the fractions eluted from Duolite C 10 column. ⩘ Yeast mannan; – – – – pH of effluent. Other symbols as in Fig. 2.

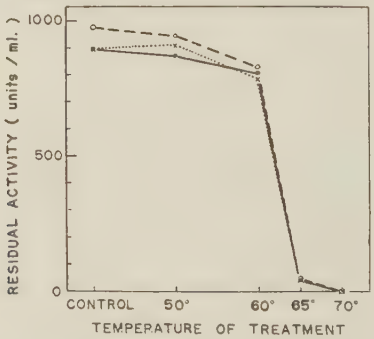

Fig. 4. Variation of three enzyme activities of the resin-eluted enzyme solution after subjection to heat treatment. Symbols as in Fig. 2.

60°, 65° and 70° respectively and cooled. After adequate dilution, the level of each of the three enzyme activities in every sample and in the untreated enzyme solution (control) were measured. The results are shown in Fig. 4, from which it can be seen that the three enzyme activities were inactivated in parallel as the temperature of treatment was raised.

Acid and Alkaline Treatment—Three ml. of the resin-eluted enzyme solution was put into each of eight tubes, the pH adjusted to various values with *N* HCl or NaOH as noted in Fig. 5 and allowed to stand at 0°. After 8 days, each solution was readjusted to pH 4.0, made up to 6 ml. and the three enzyme activities measured. As shown in Fig. 5, all activity ratios were essentially constant, although the absolute values of the activity were lowered according to the pH of treatment. It can also be seen from Fig. 5 that the amylase of this yeast tolerates an acid medium relatively better than an alkaline.

Paper Electrophoresis—An apparatus for continuous paper electrophoresis of preparative type (*6*) (Mitamura Co., Ltd.) was used. *M*/50 barbiturate buffer of pH 8.1 was allowed to flow down along a curtain of filter paper (Toyo Roshi, No. 131) as the supporting medium and a potential of 700 volts was applied across the platinum wire electrodes, giving an observed current of 12 milliamperes. The crude enzyme solution, well dialyzed against a separate

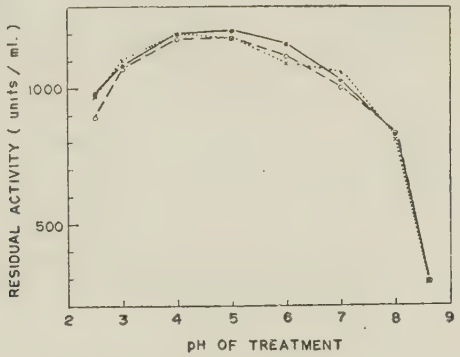

Fig. 5. Variation of three enzyme activities of the resin-eluted enzyme solution after subjection to acid or alkaline treatment. Symbols as in Fig. 2.

portion of the supporting buffer, was fed in by a 2 mm. wide filter paper wick at the center of the curtain. After 50 hours' flow, the collecting tubes were removed from the apparatus and the contents of each pair of neighboring tubes were pooled to make 24 tubes in all. The enzyme activities of all fractions were measured and the results are illustrated in Fig. 6. It can be clearly seen from Fig. 6 that distribution curves for the enzyme activities are practically overlapping and that their peaks are located at the same fraction number, although all are displaced toward the anode. It can also be seen from Fig. 6 that yeast mannan migrated less than the enzyme.

Variation of Carbon Sources for Growing the Yeast—The yeast cells were grown in media containing eight different carbon sources and the corresponding eight crude enzyme solutions were prepared by the procedure described under "methods." Each enzyme solution was diluted in proportion to the wet weight of the cells harvested and the three enzyme activities were measured. The results are summarized graphically in Fig. 7. As shown by the figure,

the amount of the enzyme formed in yeast cells was markedly influenced by the carbon source used for growth, the ratio between highest and lowest amounts being over 100 : 1, whereas the ratio of three enzyme activities was essentially identical in each enzyme solution. It is noteworthy that the enzyme activities of those three enzyme solutions, which were prepared from

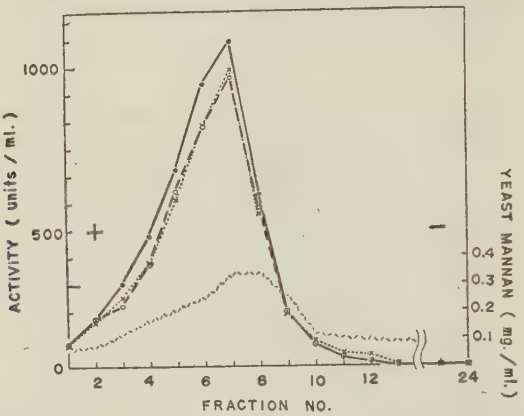

Fig. 6. Distribution of three enzyme activities and yeast mannan in the fractions obtained electrophoretically from the crude enzyme solution. Yeast mannan. Other symbols as in Fig. 2.

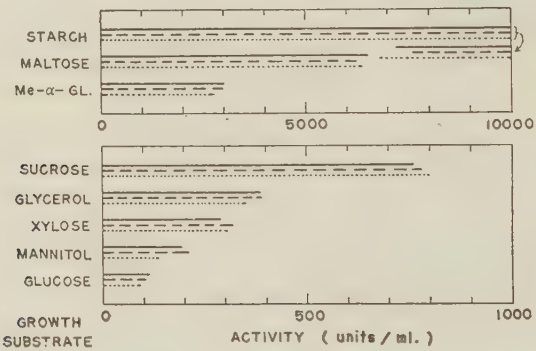

Fig. 7. Three enzyme activities of eight sorts of enzyme solutions obtained from the cells which were grown in media containing different carbon sources. Symbols as in Fig. 2.

cells grown in media containing starch, maltose and methyl α-glucoside, all postulated to be substrates of the amylase in question, are remarkably higher than those of the other enzyme solutions.

DISCUSSION

Several amylases possessing maltase activity have so far been found in molds, but their heterosidase or transglucosylase activity has been denied

(*7*, *8*) or not described (*9–11*). As for the amylase of *Candida tropicalis var. japonica*, it can in all probability be concluded from the experimental results obtained above which show that amylase, maltase and transglucosylase activities varied essentially in parallel not only on various treatments of the enzyme preparation from this yeast but also in various enzyme solutions obtained from the cells under different growth conditions, that these three enzyme activities belong to a single enzyme protein. It need not be said that this amylase can hydrolyze phenyl α-glucoside and its ability to hydrolyze methyl α-glucoside is clear from the previous paper (*1*). Thus, the amylase of this *Candida* yeast exceeds all previously known ones in the breadth of its specificity. However, considering the existence of amylases which can hydrolyze both starch and maltose (*7–11*) and of α-glucosidases which can hydrolyze various hetero α-glucosides besides maltose (*12*), it is not unreasonable to suppose that the *Candida* amylase can hydrolyze all these substrates which share the possession of α-glucosyl groups in common. Further it is probable, as Morton (*13*) indicated, that hydrolases characterized by broad substrate specificity can carry out group transfer and evidence is actually presented by the above experiements for possession of the power of transglucosylation by the *Candida* amylase.

Among the polysaccharide-hydrolyzing enzymes, only a few have hitherto been proved to carry out group transfer: the inulinase of *Helianthus tuberosus* (*14*) and Takadiastase (*15*), testicular hyaluronidase (*16*) and *Irpex* cellulase, the transferring action of which has recently been noted by Nishizawa.* The *Candida* amylase follows them. A great many amylases derived from various sources have been studied up to now but none of them has been discussed in this respect, although transglycosylase activity of any amylase would not be absolutely improbable. However, the *Candida* amylase might well be regarded as a peculiar case for the present from the fact that the hydrolysis product produced by this amylase from starch exhibited falling mutarotation in distinction from other amylases of the saccharifying type.** α-glucosyl transfer from starch to various acceptors other than water is now the subject of an investigation, which will be published in the near future.

As demonstrated in a previous paper (*1*), the *Candida* amylase (acid α-glucosidase), in spite of its broad specificity, has no hydrolyzing activity towards sucrose and probably none towards trehalose. It is, however, of interest that in these compounds the α-glucosyl group is bound to the carbonyl groups of their aglyconic sugars.

SUMMARY

Amylase of *Candida tropicalis var. japonica* was inferred to have maltase

* Nishizawa, K., Unpublished observations. Further, Dr. M.A. Jermyn said to the author the most recent that he got some indications that cellulase of *Stachybotrys atra* was a transferring enzyme.

** Sawai, T., Unpublished observations.

and transglucosylase activities on the basis of the following experimental results:

Optimum pH's of these three enzyme activities were all 4.0 and activity ratios among them were always essentially constant even if the enzyme preparation was developed on an ion exchange resin column, treated with heat, acid or alkali, or fractionated by electrophoresis, or the yeast cells from which enzyme solutions were obtained were grown on different carbon sources.

The author wishes to express his hearty gratitude to Prof. T. Miwa, Prof. J. Fukumoto and Dr. T. Yamamoto for their kind advices and encouragement, and to Dr. M.A. Jermyn, Wood Textile Research Laboratory, Commonwealth Scientific and Industrial Research Organization, Australia, for his kindness in reviewing this manuscript. The major part of this work was carried out in the laboratory, Institute of Polytechnics, Osaka City University. The expenses of this work were in part defrayed by a Grant in Aid for Scientific Research from the Ministry of Education.

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STUDIES ON THE METABOLISM OF UREA AND OTHER NITROGENOUS COMPOUNDS IN CHLORELLA ELLIPSOIDEA

II. CHANGES ON LEVELS OF AMINO ACIDS AND AMIDES DURING THE ASSIMILATION OF AMMONIA AND UREA BY NITROGEN-STARVED CELLS*

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In the foregoing paper of this series of studies, reports were made on the utilization of various nitrogenous substances by *Chlorella ellipsoidea* (*1*). It was revealed that the nitrogen-starved cells of the alga were capable of assimilating a variety of nitrogenous substances such as ammonia, urea, nitrate, arginine, ornithine and citrulline, and presumably at the expense of the energy liberated by the respiratory process, which was always found to be remarkably enhanced on addition of the nitrogen sources. The purpose of the present work was to investigate the process of the assimilation in more detail. The changes that take place in the cell during the assimilation of ammonia and urea were followed with special reference to the nitrogenous constituents, amides and amino acids, involved in the soluble nitrogen fraction of the cell. Comparison was made between the results obtained with ammonia and urea as the nitrogen source. The most salient feature in common was the important part played by arginine as an intermediary in the assimilatory process in question. The metabolism of arginine was also followed in this connection. The other substances of importance in this respect were found to be the group of amides, especially in the assimilation of ammonia. The reults obtained were discussed along with the data reported by other investigators.

EXPERIMENTAL

Nitrogen-starved (heterotrophic) culture of *Chlorella ellipsoidea* used in the present work was obtained as described in the previous paper (*1*). Washed cells were suspended in a nitrogen-free salt medium** (pH 5.5–5.6). Fifty ml. of the cell suspension were shaken by tilting through an angle of 30° in a L-shaped flask (150 ml. in capacity) at 25°. After thermal equilibration, 10 ml. of solution of urea, ammonium sulfate or L-arginine, dissolved in the

* This work was partly presented at the Annual Meeting of the Botanical Society of Japan held in Hiroshima on Oct. 13, 1955.

** Composition of the nitrogen-free basal medium: KH_2PO_4, 1.25 g.; $MgSO_4 \cdot 7H_2O$, 1.25 g.; $FeSO_4 \cdot 7H_2O$, 0.003 g.; Arnon's A5 solution (*2*), 1 ml. in 1,000 ml. of distilled water; pH adjusted to 5.5–5.6 with KOH.

basal salt medium, were added. Determination of the compounds to be investigated was performed with aliquots of the suspension removed immediately after the addition and at appropriate time intervals thereafter.

Extraction of Soluble Nitrogenous Compounds—The procedure used in extracting the cellular nitrogenous compounds was essentially the same as that employed by Syrett (*3*); immediately after sampling the material (5 ml.), 0.5 ml. of 1.1 per cent trichloroacetic acid was added and the mixture heated at 70° for 15 minutes. The cells were removed by centrifugation at $1,500 \times g$ for 10 minutes and the clear extract decanted. The cells were reextracted two more times with 5 ml. of 0.1 per cent trichloroacetic acid, each time for 15 minutes at 70°. The combined extracts were made up to 25 ml. with distilled water and stored at 0°.

For the estimation of amino acids the following procedure of extraction was adopted; 2 ml. samples of the reaction mixture were withdrawn at the same intervals as above and centrifuged at $1,500 \times g$ for 5 minutes. The supernatant was decanted and saved for the estimation of the nitrogenous substances, urea, ammonia and arginine, remaining in the reaction medium, while the residue was extracted with 5 ml. of 70 per cent (*v/v*) alcohol for 15 minutes at 70°. Extraction was repeated two more times. The former extract is represented in the following as "Extract A" and the latter as "Extract B."

Estimation of Nitrogenous Compounds—(*a*) *Urea, Ammonia and Arginine in Medium*—These were measured by the method described in the previous paper (*1*): ammonia was determined with Conway's diffusion technique (*4*), urea as ammonia after urease digestion (*4*) and arginine by Sakaguchi's method (*5*).

(*b*) *Total Soluble and Insoluble Nitrogen*—The total nitrogen was determined for each 1 ml. of samples as ammonia after Kjeldahl digestion. Digestion was continued for at least 12 hours, using 1 ml. of concentrated H_2SO_4, with the addition of $HgSO_4$, K_2SO_4 mixture (1 : 1) and $K_2S_2O_8$. When the digestion was complete, the solution was diluted to 10 ml. with distilled water. The amount of ammonia in 1 ml. of this solution was determined colorimetrically at 400 mμ using the Nessler reagent after 24 hours diffusion in a Conway unit.

The *soluble* nitrogen was estimated in the same manner with 5 ml. of "Extract A." To determine the content of cellular soluble nitrogen, the amount of nitrogen in the medium was subtracted from that of the total soluble nitrogen in each case.

The difference between the total nitrogen and total soluble nitrogen was regarded as the quantity of *insoluble* nitrogen.

(*c*) *Amide Nitrogen*—The total amide nitrogen was determined by Conway's method (*4*). Two ml. of "Extract A" were digested for 3 hours at 100° with 1 ml. of 3 *N* H_2SO_4. After digestion, the ammonia was determined and compared with that originally present, and the increase of ammonia nitrogen was taken as the total amide nitrogen. Unstable amide nitrogen (glutamine) was determined by the method of Vickery *et al.* (*6*). The difference between this and the total amide nitrogen was regarded as the amount of stable amide nitrogen (asparagine). When urea was used as the nitrogen source, correction for the amount of ammonia produced by a decomposition of urea during the digestion was made with the value obtained from the control run carried out with the same amount of urea. Urea nitrogen was determined colorimetrically with 1 ml. of "Extract A" by Fearon's method modified by Knivett (*7*, *8*).

(*d*) *Total Free Amino Nitrogen*—Using 2 ml. each of "Extract A" total free amino nitrogen was estimated by the method of Russell *et al.* (*9*). To prevent the interference of free ammonia, the sample was left overnight with *N* NaOH *in vacuo* in a sulfuric acid dessicator. The alkali was then carefully neutralized and the determination of amino-nitrogen carried out. The intensity of the color developed wes measured electrophotometrically at 470 mμ. Calibration was made against an equimolar mixture of alanine and glutamic acid (*3*) and all results were expressed on this basis.

(*e*) *Amino Acid Nitrogen*—"Extract B" was evaporated *in vacuo* almost to dryness and diluted to 0.5 ml. with distilled water. For each 0.2 ml. of this solution, the α-amino nitrogen of individual amino acids was determined, using the quantitative paper chromatographic method (*10*). Each amino acid separated as a spot during 24 hours development on Toyo No. 50 filter paper, a phenol-ammonia-water mixture (80 : 1 (28 per cent) : 20) being used as the solvent. The amino acid eluted from each spot was colored with ninhydrin (*11*) and determined spectrophotometrically at 570 mμ. Among the amino acids which were detectable paper-chromatographically (glutamic acid, aspartic acid, alanine, glycine, arginine, valine, leucine and proline) quantitative determination with this method was carried out only for glutamic acid, aspartic acid, alanine and glycine. Paper chromatographic separation being inadequate for amino acids such as arginine, ornithine and citrulline, these were respectively estimated colorimetrically with aliquots of "Extract A" by the following methods: *i.e.*, Sakaguchi's method for arginine (*5*), Chinard's for ornithine (*12*) and Knivett' for citrulline (*8*). Since the amount of other amino acids (valine and leucine) was very meager, no attempt was made for determining these compounds.

The colorinetry was carried out with a Beckman spectrophotometer, Model DU, throughout the present work except for that of arginine, for which a simple photometer, using a selenium photovoltatic cell and an appropriate filter, was employed.

RESULTS

Changes in Cellular Composition during the Assimilation of Ammonia—When ammonium sulfate is added to nitrogen-starved cells of *Chlorella*, ammonia is rapidly assimilated and organic nitrogenous compounds are formed. A typical result is shown in Fig. 1. After 3 hours, about 60 per cent of the assimilated ammonia is present in the cells in *soluble* form. The remainder has been converted to *insoluble* compounds, no trace amounts of free ammonia being ever detected in the cell.

The increase in *soluble* nitrogen fraction is largely due to amide, chiefly glutamine, in the first stage. A steady increase of *free amino* nitrogen was also observed. Among the amino acids tested, arginine showed the most marked increase. Increase was also observed in the amounts of ornithine and acidic amino acids, glutamic and aspartic acids, but this was very small. No net formation was found in citrulline and urea.

Changes during the Assimilation of Urea—A typical result for these is presented in Fig. 2. During the first 4 hours, almost all the nitrogen of the assimilated urea was present in *soluble* form; it was then gradually incorporated into *insoluble* compounds. Since no net accumulation of urea in the cells was ever observed, it may be assumed that urea was taken up at a rate not in surplus to its further conversion into other nitrogenous compounds. The amount of amide nitrogen shows only a small and gradual increase during urea assimilation in contrast with the rapid increase found in the case of ammonia assimilation.

Although an increase of such amino acids as arginine and alanine was also observed, the amino acid showing the greatest increase was, as in the case of ammonia assimilation, arginine. The rate of formation of arginine, however, was more rapid and the induction period, which was always noticed in the case of ammonia assimilation, was never observed. No change of the

content of acidic amino acid occurred during the examined period.

Changes during the Assimilation of Arginine—In connection with the material accumulation of arginine observed during the assimilation of both ammonia and urea, the process of arginine assimilation were also examined. The

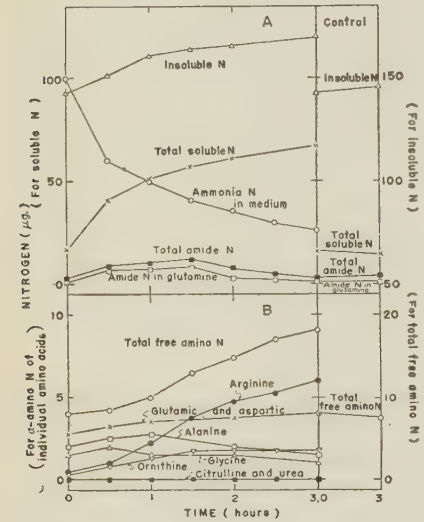

Fig. 1. Changes of nitrogenous composition during the assimilation of ammonia by nitrogen-starved cells of *Chlorella*. Initial concentration of ammonium sulfate; 100 μg. ammonia-N per ml. (Control without the addition of N-source). Condition of incubation; pH 5.6; 25°; aerobic; in the dark (6.2 mg. dry weight cells per ml.). All figures refer to 1 ml. of suspension.

(A) Changes in total *soluble*, *insoluble* and amide nitrogen fractions and in ammonia in the medium (μg.).

(B) Changes in α-amino-N of total free amino acid and of individual amino acids and N of urea (μg.).

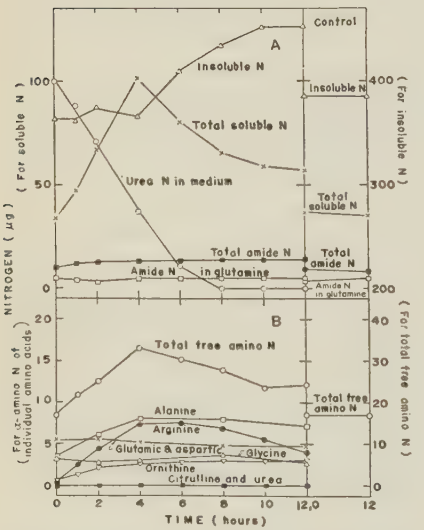

Fig. 2. Changes of nitrogenous composition during the assimilation of urea by nitrogen-starved cells of *Chlorella*. All experimental conditions were the same as in Fig. 1. except for the addition of urea (100 μg-N per ml.) instead of ammonium sulfate (17.4 mg. dry weight cells per ml.).

results are shown in Figs. 3 and 4. From Fig. 3 it may be seen that a large part of the assimilated arginine is accumulated temporarily in the cells; the remainder may undergo some sort of conversion or decomposition. After the exhaustion of the exogenous arginine, the free arginine in the cells is gradually

converted to other compounds (Fig. 4). In fact the amount of amide nitrogen showed an increase, which was found to be due to a stable amide (asparagine),

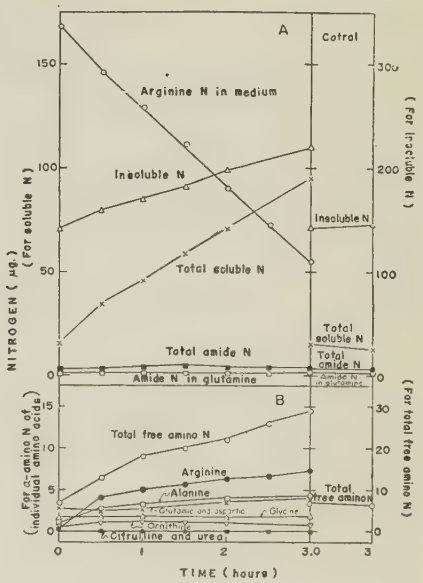

FIG. 3. Changes of nitrogenous composition during the assimilation of arginine by nitrogen-starved cells of *Chlorella*. All experimental conditions were the same as in Fig. 1 except for the addition of L-arginine (170 μg.-N per ml.) instead of ammonium sulfate. (6.0 mg. dry weight cells per ml.).

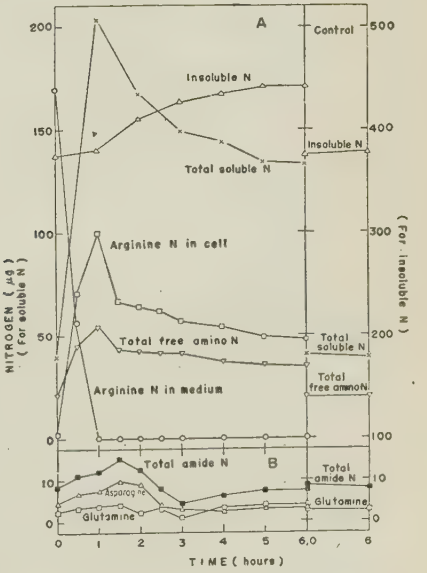

FIG. 4. Changes of nitrogenous composition during the assimilation of arginine by nitrogen-starved cells of *Chlorella*. All experimental conditions were the same as in Fig. 3. (18.5 mg. dry weight cells per ml.).
(A) Changes in total *soluble*, *insoluble* and free amino nitrogen (μg.).
(B) Changes in amide nitrogen (μg.).

at the first stage, The fraction was then subject to a gradual decay until a level as low as the original one was reached. No production of free ammonia was detected during these changes. Increase in amino acids other than

arginine was also observed, but this was very small and gradual except in the case of alanine.

DISCUSSION

A temporary and no less marked increase in *total soluble* nitrogen accompanied by a gradual and steady rise in *insoluble* nitrogen of the cell, represents the general feature of events occurring when nitrogenous substances such as ammonia, urea and arginine were administrated to the nitrogen-starved cells of *Chlorella*, indicating the effective utilization of the substances as sources of nitrogen. Although the whole experimental results were similar in this respect, there were certain differences noticed among the different nitrogen sources so far as the rises and falls of individual substances involved were concerned.

Temporary accumulation of the added nitrogen source inside the cell was found to take place only with arginine. A considerable portion of the substance was actually discovered in the *soluble* nitrogen fraction of the cell before it was gradually converted into other nitrogenous substances. That this was never the case with ammonia and urea as nitrogen source, may in some was be connected with the known injurious action of these substances towards living cells.

Among various substances to be recovered in the *soluble* fraction, important roles as the intermediary of the assimilatory process in question must be ascribed to those that show appreciable concentration changes during the course of assimilation. Among these were found the basic amino acid, arginine, and the amides such as glutamine and asparagine.

The importance of amides as intermediates in the assimilation of ammonia has already suggested by other investigators. Syrett (*3*), working with nitrogen-starved cells of *Chlorella vulgaris*, observed a temporary increase of the amide fraction (mostly glutamine) during the rapid assimilation of ammonia. In his experiments with barley roots, and using heavy nitrogen as a tracer, Yemm (*13*) presented evidence for the primary synthesis of glutamine from ammonia, followed by its secondary conversion into asparagine and various amino acids. Similar results have been obtained with nitrogen-starved yeast cells (*14*). The results of the present study also seem to be in accord with this line of inference. With urea as the source of nitrogen, however, no appreciable increase in amide nitrogen was noticed. By far more pronounced in this case was the transient appearance of arginine in the course of assimilation (Fig. 2). Therefore, at least in the case of urea assimilation, the primary intermediate may be arginine. It may be noticed in this connection that in ammonia assimilation (Fig. 1) arginine appears always after a certain time lag, indicating that arginine is formed from ammonia in a roundabout way as compared with the process of arginine formation from urea. An induction period in the formation of arginine (estimated as *basic amino acids*) as preceded by the transient appearance of amide fraction is evidently seen also in the data presented by Syrett pertaining to the

ammonia assimilation of nitrogen-starved cells of *Chlorella* (*15*).

In view of the various data presented above, it seems justifiable to assume that the assimilation of ammonia and urea would proceed in the following manner: assimilation of ammonia:

ammonia ⟶ amide (glutamine) ⟶ arginine ⟶ cell substance

assimilation of urea:

urea ⟶ arginine ⟶ cell substance

The importance of arginine in the nitrogen metabolism has been suggested also by other investigators. Syrett and Fowden (*15*), for instance, showed that the basic amino acids (arginine, lysine and ornithine) represented a large propotion of the free amino acids formed in the first stage of ammonia assimilation in *Chlorella vulgaris*. Once arginine is formed in the cell, or taken up from the surrounding medium as in the experiment shown in Fig. 4, there seem to be various possible ways of mutual conversion between the amino acid and amides (asparagine and glutamine), as suggested by the experimental results. As to the details of the sequence of events in the synthesis of arginine from ammonia or urea, we have at present no partinent information. The possibility that urea is assimilated via ammonia has been excluded beyond any doubt by the data previously reported (*1*), namely: (*a*) that there was no trace of urease activity detectable in *Chlorella* cells, and (*b*) that no trace of ammonia could be detected during the precess of urea assimilation. Although there seems to be certain probability for Krebs' ornithine cycle operating in the process of urea assimilation, the present study could procure no conclusive evidence for it except that the intermediary formation of ornithine, one of the members of the cycle, was always observed during the incubation of the algal cells with ammonia, urea as well as arginine as the source of nitrogen. The addition of ornithine in the reaction mixture, however, caused no acceleration whatever of the assimilatory process. Test for the other members of cycle in the reaction mixture (*i.e.*, citrulline, arginine) also gave negative results: no accelerating or sparking effect of these substances in question was ever discovered.

More or less marked changes in the levels of simpler amino acids, espesially those of alanine and glycine, were sometimes observed, but the data at hand do not permit of a decision as to whether these substances represent intermediates in the alternative main pathway or products formed only in the bypath in the nitrogenous metabolism of *Chlorella* cells.

SUMMARY

1. The process of assimilation of ammonia and urea added as the sole source of nitrogen was investigated with nitrogen-starved cells of *Chlorella ellipsoidea*.

2. The changes that occurred during the assimilation were followed with special reference to the levels of various amino acids and amides involved in the *soluble* nitrogen fraction of the cell, and the results obtained with ammonia were compared with those obtained with urea.

3. Tentative formulae were proposed for the pathway of assimilation of the nitrogen souces as follows:

assimilation of ammonia: ammonia → amide (mainly glutamine) → arginine → cell substance.

assimilation of urea: urea → arginine → cell substance.

4. Additional experimental data showing the role of arginine in the assimilatory processes in question were presented.

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